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PROTEINS, GENES AND THEIR USE FOR DIAGNOSIS AND TREATMENT OF CARDIAC RESPONSE

1. INTRODUCTION

The present invention relates to the identification of proteins and protein isoforms that are associated with cardiac response to xenobiotics, including its onset and development, and of genes encoding the same, and to their use for clinical screening, diagnosis, prognosis, therapy and prophylaxis, as well as for drug screening and drug development.

2. BACKGROUND OF THE INVENTION

The heart's role in circulating molecules and cells throughout the body is not just that of a passive pump. The heart cells also actively responds to these molecules and cells, which are defined herein as "effector agents," for example by rapidly modulating metabolic pathways and by expressing new patterns of protein export and cell surface expression. Any disruptions in cardiac function can lead to serious, often life-threatening, consequences. A wide variety of effector agents are provided herein as examples of, but are not limited to, inducers of cardiac responses:

Endogenous effector agents

- ischemia
- thrombus
- diabetes
- hypercholesterolemia
- hypertension
- hypotension
- physical inactivity
- poor nutrition
- overweightness / obesity
- Genetic disease
    - familial cardiomyopathy
    - familial valvular dysplasia
    - glycogen storage disease
    - muscular dystrophy
- Marfan syndrome

Exogenous Effector Agents

· Xenobiotics

· Chemical agents and medications

- nicotine & smoking
- chemotherapeutics (e.g., a variety of approved and experimental treatments for indications such as cancer)
- coagulants and anticoagulants
- vasoconstrictors and vasodialators
- drugs of abuse (e.g., cocaine, amphetamines)
- performance enhancing drugs (e.g., steroids, testosterone)

· Trauma

- accidental
- surgery

The heart is architecturally complex and composed of many unique cell types. Heart-affecting effector agents may exclusively affect just one of these cell types, or, more commonly, may interfere with several types simultaneously. Thus, affected areas may range from highly focal to diffuse lesions, and may spread or refocus over time. A wide variety of cardiac changes are defined herein as examples of, but not limited to, cardiac responses to effector agents:

· Aneurysm

· Angina

· Arrhythmia

· Cardiomyopathy (dilated, hypertrophic, restrictive)

· Cardiac arrest (myocardial infarction)

· Cor pulmonale

· Coronary artherosclerosis

· Edema

· Endocarditis (acute bacterial, prosthetic valvular, right-sided, infective)

· Hemorrhage

· Mitral valve prolapse syndrome

· Murmur

· Paricarditis

· Shock (hypovolemia, vasodilation, septic, cardiogenic)

· Stenosis

The following list outlines currently validated measures of cardiac responses to one or more of the efector agents described above:

· Nonintrusive assays

- blood pressure monitoring
- exercise / stress test
- soft tissue imaging (e.g., doppler flow ultrasonography, magnetic resonance imaging, computed tomography, positron emission tomography)
- radiography (e.g., frontal / leteral chest x-ray)
- radionuclide imaging (e.g., myocardial perfusion, SPECT, ventriculography
- echocardiography
- assessment of cardiac components in blood (e.g. troponin, myoglobin, and cardiolipin)
- serum troponin determination

· Intrusive assays

- catheterization (e.g., peripheral, central, arterial, cardiac)
- angiocardiography
- surgery

All of these current measures of cardiac response suffer from one or more significant limitations. For example, the non-intrusive assays show poor correlation with cardiac histopathology and generally provide no prospective measure of how the cardiac response will change over time. The intrusive cardiac response assays also suffer from the limitation that they present significant risk to the test subject. Therefore, they generally are not employed unless the subject's life is already under serious threat. In addition, the intrusive assays require time-consuming and costly interpretation by expert pathologists, and may also provide ambiguous results if the tissue changes are not homogeneous across the sample examined.

The current measures of cardiac response are also severely limited in their usefulness in facilitating the development of new treatments for human disease.

Some anecdotal studies have shown that the levels of certain heart cell or blood proteins change in response to exposure to effector agents. However, we are unaware of any of these studies systematically encompassing both e.g. (i) effects of xenobiotic treatments over several time points or (ii) assessing the progression of

response to xenobiotics, in order to identify statistically significant changes in protein levels.

Due to the costly and time consuming nature of existing, often ambiguous, tests it would be highly desirable to measure a substance or substances in samples of blood or heart cells that would lead to a positive diagnosis of cardiac response or that would help to exclude cardiac response from a differential diagnosis.

The development of new pharmaceutical compositions and/or treatment regimens directed towards the treatment or prophylaxis of diseases, infectious or otherwise, relies heavily on the ability to screen candidate compounds for possible toxic or pathological responses, e.g. vascular response. In drug development, a putative drug is tested in a battery of assays and in laboratory animals to ascertain its safety (i.e. lack of toxicity) and effectiveness. The costs associated with the development of new pharmaceutical reagents are ever increasing, particularly when new compositions enter clinical trials. It is not unheard of for promising pharmaceutical candidates to pass the appropriate laboratory tests and enter the expensive stage of animal and human clinical trials, only to present toxic or pathologic effects in the *in vivo* setting for the targeted patient, normally humans. The elimination of previously-promising drug candidates at such a late stage in product development is a major factor in the high costs of new effective drugs which ultimately do pass the final clinical trials.

Therefore, a need exists to identify cardiac response-associated proteins as sensitive and specific biomarkers for the diagnosis, to assess severity and predict the outcome of cardiac response in living subjects, and, in particular, to allow the screening of drug candidates for their ability to induce a vascular response. Additionally, there is a clear need for new therapeutic agents for cardiac response that work quickly, potently, specifically, and with fewer side effects.

3. SUMMARY OF THE INVENTION

The present invention provides methods and compositions for clinical screening, diagnosis, prognosis, therapy and prophylaxis of cardiac response, in particular, the screening of drug candidates for their ability to induce a cardiac response; for monitoring the effectiveness of cardiac response treatment; for selecting participants in clinical trials; for identifying patients most likely to respond

to a particular therapeutic treatment and for screening and development of drugs for treatment of cardiac response.

A first aspect of the invention provides methods for diagnosis of cardiac response that comprise analyzing a sample of body fluid, *e.g.,* blood, by two-dimensional electrophoresis to detect the presence or level of at least one Cardiac Response-Associated Feature (CRF), *e.g.*, one or more of the CRFs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, for drug screening and development, and identification of new targets for drug treatment.

A second aspect of the invention provides methods for diagnosis of cardiac response that comprise detecting in a sample of body fluid, *e.g.,* blood, the presence or level of at least one Cardiac Response-Associated Protein Isoform (CRPI), *e.g.*, one or more of the CRPIs disclosed herein or any combination thereof. These methods are also suitable for clinical screening, prognosis, monitoring the results of therapy, identifying patients most likely to respond to a particular therapeutic treatment, drug screening and development, and identification of new targets for drug treatment.

A third aspect of the invention provides antibodies, *e.g.* monoclonal and polyclonal antibodies capable of immunospecific binding to a CRPI, *e.g.*, a CRPI disclosed herein.

A fourth aspect of the invention provides a preparation comprising an isolated CRPI, *i.e.*, a CRPI free from proteins or protein isoforms having a significantly different isoelectric point or a significantly different apparent molecular weight from the CRPI.

A fifth aspect of the invention provides kits that may be used in the above recited methods and that may comprise single or multiple preparations, or antibodies, together with other reagents, labels, substrates, if needed, and directions for use. The kits may be used for diagnosis of disease, or may be assays for the identification of new diagnostic and/or therapeutic agents.

A sixth aspect of the invention provides methods of treating cardiac response, comprising administering to a subject a therapeutically effective amount of an agent that modulates (*e.g.*, upregulates or downregulates) the expression or activity (*e.g.* enzymatic or binding activity), or both, of a CRF, or a CRPI in subjects

having cardiac response, in order to prevent or delay the onset or development of cardiac response, to prevent or delay the progression of cardiac response, or to ameliorate the symptoms of cardiac response.

A seventh aspect of the invention provides methods of screening for agents that modulate (*e.g.*, upregulate or downregulate) a characteristic of, *e.g.*, the expression or the enzymatic or binding activity, of a CRF, a CRPI, a CRPI analog, or a CRPI-related polypeptide. This aspect of the invention being particularly useful in determining the ability of drug candidiates to induce a cardiac response.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a flow chart depicting the characterization of a CRF and the relationship of a CRF to a CRPI. A CRF may be further characterized as or by a CRPI having a particular peptide sequence associated with its pI and MW. As depicted herein, a CRF may comprise one or more CRPIs, which have indistinguishable pI and MWs using the Preferred Technology, but which comprise distinct peptide sequences. The peptide sequence(s) of the CRPI can be utilized to search database(s) for previously-identified proteins comprising such peptide sequence(s). In some instances, it can be ascertained whether a commercially-available antibody exists which may recognize the previously identified protein and/or a variant thereof. It should be noted that the CRPI may correspond to the previously-identified protein, be a variant of the previously identified protein, or be a previously unknown protein

Figure 2 is an image obtained from 2-dimensional electrophoresis of rat serum, which has been annotated to identify thirteen landmark features, designated F1 to F13, and which are illustrative of an embodiment of an aspect of the present invention.

5. DETAILED DESCRIPTION OF THE INVENTION

The invention described in detail below provides methods and compositions for clinical screening, diagnosis and prognosis of cardiac response in a mammalian subject for identifying patients most likely to respond to a particular therapeutic treatment, for monitoring the results of cardiac response therapy, for drug screening

and drug development. When the invention is used to determine the ability of drug candidiates to induce a cardiac response, the body fluid which is analysed for the presence or level of at least one cardiac response feature is preferably from a non-human mammal. The non-human mammal is preferably one in which the induction of a cardiac response by endogenous and/or exogenous effector agents is predictive of the induction of such a response in humans. The rat is a particularly suitable mammal for use in this aspect of the invention.

The invention also encompasses the administration of therapeutic compositions to a mammalian subject to treat or prevent cardiac response. The mammalian subject may be a non-human mammal, but is preferably human, more preferably a human adult, i.e. a human subject at least 21 (more preferably at least 35, at least 50, at least 60, at least 70, or at least 80) years old. For clarity of disclosure, and not by way of limitation, the invention will be described with respect to the analysis of blood samples. However, as one skilled in the art will appreciate, the assays and techniques described below can be applied to other types of samples, including a body fluid (*e.g.* spinal fluid, plasma, saliva or urine), a tissue sample from a subject at risk of having or developing cardiac response (*e.g.* a biopsy such as a cardiac biopsy) or homogenate thereof. The methods and compositions of the present invention are useful for screening, diagnosis and prognosis of a living subject, but may also be used for postmortem diagnosis in a subject, for example, to identify family members of the subject who are at risk of developing the same disease.

The following definitions are provided to assist in the review of the instant disclosure.

5.1. Definitions

"Cardiac Response" refers to and includes the activities and consequent alterations in cardiac function and other organ or cellular function and any condition that comes about from interaction of the heart with endogenous or exogenous effector agents, including xenobiotics, viruses or other biological agents, particularly those agents which are cardiotoxic, which can generate a cardiac response or which otherwise reduce or alter the function or physiological response of the heart. Cardiac response encompases and includes those activites, alterations and

physiological occurrences in the heart, or otherwise associated with the heart or the heart's function, which take place during any alteration of the heart including but not limited to any aspect or phase of aneurysm, angina, arrhythmia, cardiomyopathy (dilated, hypertrophic, restrictive), cardiac arrest (myocardial infarction), cor pulmonale, coronary artherosclerosis, edema, endocarditis (acute bacterial, prosthetic valvular, right-sided, infective), hemorrhage, mitral valve prolapse syndrome, murmur, paricarditis, shock (hypovolemia, vasodilation, septic, cardiogenic), stenosis. The effector agents include but are not limited to ischemia, thrombus, diabetes, hypercholesterolemia, hypertension, hypotension, physical inactivity, poor nutrition, overweightness/obesity, genetic disease, trauma, chemical agents, and medications and xenobiotics.

"Feature" refers to a spot detected in a 2D gel, and the term " Cardiac Response- Associated Feature" (CRF) refers to a feature that is differentially present in a sample from a subject having a Cardiac Response compared with a sample from a subject free from a Cardiac Response. A feature or spot detected in a 2D gel is characterized by its isoelectric point (pI) and molecular weight (MW) as determined by 2D gel electrophoresis, particularly utilizing the Preferred Technology described herein. As used herein, a feature is "differentially present" in a first sample with respect to a second sample when a method for detecting the said feature (*e.g.*, 2D electrophoresis) gives a different signal when applied to the first and second samples. A CRF, (or a protein isoform, i.e. CRPI, as defined *infra*) is "increased" in the first sample with respect to the second if the method of detection indicates that the CRF, or CRPI is more abundant in the first sample than in the second sample, or if the CRF, or CRPI is detectable in the first sample and substantially undetectable in the second sample. Conversely, a CRF, or CRPI is "decreased" in the first sample with respect to the second if the method of detection indicates that the CRF, or CRPI is less abundant in the first sample than in the second sample or if the CRF, or CRPI is undetectable in the first sample and detectable in the second sample.

Preferably, the relative abundance of a feature in two samples is determined in reference to its normalized signal, in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, *e.g.*, (a) to the total protein in the sample being analyzed (*e.g.*, total protein loaded onto a gel); (b) to an Expression Reference Feature (ERF) i.e., a

feature whose abundance is substantially invariant, within the limits of variability of the Preferred Technology, in the population of subjects being examined, *e.g.* the ERFs disclosed below, or (c) more preferably to the total signal detected as the sum of each of all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

"Fold change" includes "fold increase" and "fold decrease" and refers to the relative increase or decrease in abundance of a CRF or the relative increase or decrease in expression or activity of a polypeptide (*e.g.* a CRPI, as defined *infra.*) in a first sample or sample set compared to a second sample (or sample set). A CRF or polypeptide fold change may be measured by any technique known to those of skill in the art, albeit the observed increase or decrease will vary depending upon the technique used. Preferably, fold change is determined herein as described in the Examples *infra*.

"Cardiac Response-Associated Protein Isoform" (CRPI) refers to a protein isoform that is differentially present in a sample from a subject having a Cardiac Response compared with a sample from a subject free from any Cardiac Response or that is differentially present in a sample from a subject having one or more particular Cardiac Response compared with a sample from a subject free from such one or more particular Cardiac Response or having a distinct Cardiac Response. As used herein, a CRPI is "differentially present" in a first sample with respect to a second sample when a method for detecting the said feature, (*e.g.*, 2D electrophoresis or immunoassay) gives a different signal when applied to the first and second samples (refer to CRF definition).

A CRPI is characterised by one or more peptide sequences of which it is comprised, and by further reference to a pI and MW, preferably determined by 2D gel electrophoresis, particularly utilizing the Preferred Technology as described herein. Typically, CRPIs are identified or characterized by the amino acid sequencing of CRFs (Figure 1).

Figure 1 is a flow chart depicting the characterization of a CRF and the relationship

of a CRF and CRPI(s). A CRF may be further characterized as or by a CRPI having a particular peptide sequence associated with its pI and MW. As depicted herein, a CRF may comprise one or more CRPIs, which have indistinguishable pI and MWs using the Preferred Technology, but which comprise distinct peptide sequences. The peptide sequence(s) of the CRPI can be utilized to search database(s) for previously identified proteins comprising such peptide sequence(s), for which previously identified protein it can be ascertained whether a commercially-available antibody exists which may recognize the previously identified protein and/or a member of its protein family. It should be noted that the CRPI may correspond to the previously-identified protein, be a variant of the previously identified protein, or be a previously unknown protein.

"Variant" as used herein refers to a polypeptide which is a member of a family of polypeptides that are encoded by a single gene or from a gene sequence within a family of related genes and which differ in their pI or MW, or both. Such variants can differ in their amino acid composition (*e.g.* as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation, phosphorylation).

"Modulate" in reference to expression or activity of a CRPI or a CRPI-related polypeptide refers to any change, *e.g.*, upregulation or downregulation, of the expression or activity of the CRPI or a CRPI-related polypeptide. Those skilled in the art, based on the present disclosure, will understand that such modulation can be determined by assays known to those of skill in the art.

"CRPI analog" refers to a polypeptide that possesses similar or identical function(s) as a CRPI but need not necessarily comprise an amino acid sequence that is similar or identical to the amino acid sequence of the CRPI, or possess a structure that is similar or identical to that of the CRPI. As used herein, an amino acid sequence of a polypeptide is "similar" to that of a CRPI if it satisfies at least one of the following criteria: (a) the polypeptide has an amino acid sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least

85%, at least 90%, at least 95% or at least 99%) identical to the amino acid sequence of the CRPI; (b) the polypeptide is encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding at least 5 amino acid residues (more preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues) of the CRPI; or (c) the polypeptide is encoded by a nucleotide sequence that is at least 30% (more preferably, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99%) identical to the nucleotide sequence encoding the CRPI. As used herein, a polypeptide with "similar structure" to that of a CRPI refers to a polypeptide that has a similar secondary, tertiary or quarternary structure as that of the CRPI. The structure of a polypeptide can determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy.

"CRPI fusion protein" refers to a polypeptide that comprises (i) an amino acid sequence of a CRPI, a CRPI fragment, a CRPI-related polypeptide or a fragment of a CRPI-related polypeptide and (ii) an amino acid sequence of a heterologous polypeptide (i.e., a non-CRPI, non-CRPI fragment or non-CRPI-related polypeptide).

"CRPI homolog" refers to a polypeptide that comprises an amino acid sequence similar to that of a CRPI but does not necessarily possess a similar or identical function as the CRPI.

"CRPI ortholog" refers to a non-rat polypeptide that (i) comprises an amino acid sequence similar to that of a CRPI and (ii) possesses a similar or identical function to that of the CRPI. It will be appreciated that the specific CRPIs identified in the description were derived from the rat. The skilled person will recognise that in various aspects of the invention it will be necessary to substitute the rat CRPI with the CRPI ortholog from another mammal e.g. a human. CRPI orthologs can be

identified using techniques well known to those skilled in the art for example using homology searching e.g. as described below in relation to the determination of per cent identitiy of two amino acid sequences. The similarity between the CRPIs identified and their human orthologs is on average 82% (S.E.M. = 2.71) allowing for conservative substitutions (see section 5.7). It will be appreciated that in various aspects of the claimed invention, e.g. methods of treatment, it will be necessary to substitute a CRPI with a CRPI ortholog depending on the identity of the mammal to be treated.

"CRPI-related polypeptide" refers to a CRPI homolog, a CRPI analog, a variant of CRPI, a CRPI ortholog, or any combination thereof.

"Chimeric Antibody" refers to a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.)

"Derivative" refers to a polypeptide that comprises an amino acid sequence of a second polypeptide that has been altered by the introduction of amino acid residue substitutions, deletions or additions. The derivative polypeptide possesses a similar or identical function as the second polypeptide.

"Fragment" refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, or at least 250 amino acid residues) of the amino acid sequence of a second polypeptide. The fragment of a CRPI may or may not possess a functional activity of the second polypeptide.

The "percent identity" of two amino acid sequences or of two nucleic acid sequences can be or is generally determined by aligning the sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in either sequences for best

alignment with the other sequence) and comparing the amino acid residues or nucleotides at corresponding positions. The "best alignment" is an alignment of two sequences that results in the highest percent identity. The percent identity is determined by the number of identical amino acid residues or nucleotides in the sequences being compared (i.e., % identity = # of identical positions/total # of positions x 100).

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm known to those of skill in the art. An example of a mathematical algorithm for comparing two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. The NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410 have incorporated such an algorithm. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). The ALIGN program (version 2.0) which is part of the GCG sequence alignment software package has incorporated such an algorithm. Other algorithms for sequence analysis known in the art include ADVANCE and ADAM as described in Torellis and Robotti (1994) Comput. Appl. Biosci., 10 :3-5; and FASTA described in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search.

"Diagnosis" refers to diagnosis, prognosis, monitoring, characterizing, selecting patients, including participants in clinical trials, and identifying patients at risk for or having a particular disorder or those most likely to respond to a particular therapeutic treatment, or for assessing or monitoring a patient's response to a particular therapeutic treatment.

"Treatment" refers to therapy, prevention and prophylaxis and particularly refers to the administration of medicine or the performance of medical procedures with respect to a patient, for either prophylaxis (prevention) or to cure the infirmity or malady in the instance where the patient is afflicted.

"Agent" refers to all materials that may be used to prepare pharmaceutical and diagnostic compositions, or that may be compounds, nucleic acids, polypeptides, fragments, isoforms, variants, or other materials that may be used independently for such purposes, all in accordance with the present invention.

"Blood" as used herein includes serum and plasma. As used herein, the term "serum" refers to the supernatant fluid produced by clotting and centrifugal sedimentation of a blood sample. As used herein, the term "plasma" refers to the supernatant fluid produced by inhibition of clotting (for example, by citrate or EDTA) and centrifugal sedimentation of a blood sample.

5.2 Cardiac Response-Associated Features (CRFs)

In one aspect of the invention, two-dimensional electrophoresis is used to analyze blood from a subject, preferably a living subject, in order to detect or quantify the expression of one or more Cardiac Response-Associated Features (CRFs) for screening, prevention or diagnosis of cardiac response, to determine the prognosis of a subject having cardiac response, to monitor progression of cardiac response, to monitor the effectiveness of cardiac response therapy, for identifying patients most likely to respond to a particular therapeutic treatment, or for drug development and, in particular, to determine the potential for drug candidaites to induce a vascular response.

By way of example and not of limitation, using the Preferred Technology, a number of samples from subjects having a cardiac response and samples from subjects free from a cardiac response are separated by two-dimensional

electrophoresis, and the fluorescent digital images of the resulting gels are matched to a chosen representative primary master gel image. This process allows any gel feature, characterised by its pI and MW, to be identified and examined on any gel of the study. In particular, the amount of protein present in a given feature can be measured in each gel; this feature abundance can be averaged amongst gels from similar samples (e.g. gels from samples from subjects having a cardiac response). Finally, statistical analyses can be conducted on the thus created sample sets, in order to compare 2 or more sample sets to each other.

As used herein, "two-dimensional electrophoresis" (2D-electrophoresis) means a technique comprising isoelectric focusing, followed by denaturing electrophoresis; this generates a two-dimensional gel (2D-gel) containing a plurality of separated proteins. Preferably, the step of denaturing electrophoresis uses polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Especially preferred are the highly accurate and automatable methods and apparatus ("the Preferred Technology") described in International Application No. 97GB3307 (published as WO 98/23950) and in U.S. Patent No.6,064,754, each of which is incorporated herein by reference in its entirety with particular reference to the protocol at pages 23-35. Briefly, the Preferred Technology provides efficient, computer-assisted methods and apparatus for identifying, selecting and characterizing biomolecules (*e.g.* proteins, including glycoproteins) in a biological sample. A two-dimensional array is generated by separating biomolecules on a two-dimensional gel according to their electrophoretic mobility and isoelectric point. A computer-generated digital profile of the array is generated, representing the identity, apparent molecular weight, isoelectric point, and relative abundance of a plurality of biomolecules detected in the two-dimensional array, thereby permitting computer-mediated comparison of profiles from multiple biological samples, as well as computer aided excision of separated proteins of interest.

A preferred scanner for detecting fluorescently labeled proteins is described in WO 96/36882 and in the Ph.D. thesis of David A. Basiji, entitled "Development of a High-throughput Fluorescence Scanner Employing Internal Reflection Optics and Phase-sensitive Detection (Total Internal Reflection, Electrophoresis)", University of Washington (1997), Volume 58/12-B of Dissertation Abstracts International, page 6686, the contents of each of which are incorporated herein by

reference. These documents describe an image scanner designed specifically for automated, integrated operation at high speeds. The scanner can image gels that have been stained with fluorescent dyes or silver stains, as well as storage phosphor screens. The Basiji thesis provides a phase-sensitive detection system for discriminating modulated fluorescence from baseline noise due to laser scatter or homogeneous fluorescence, but the scanner can also be operated in a non-phase-sensitive mode. This phase-sensitive detection capability would increase the sensitivity of the instrument by an order of magnitude or more compared to conventional fluorescence imaging systems. The increased sensitivity would reduce the sample-preparation load on the upstream instruments while the enhanced image quality simplifies image analysis downstream in the process.

A more highly preferred scanner is the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK), which is a modified version of the above described scanner. In the Apollo 2 scanner, the gel is transported through the scanner on a precision lead-screw drive system. This is preferable to laying the glass plate on the belt-driven system that is described in the Basiji thesis, as it provides a reproducible means of accurately transporting the gel past the imaging optics.

In the Apollo 2 scanner, the gel is secured against three alignment stops that rigidly hold the glass plate in a known position. By doing this in conjunction with the above precision transport system, the absolute position of the gel can be predicted and recorded. This ensures that co-ordinates of each feature on the gel can be determined more accurately and communicated, if desired, to a cutting robot for excision of the feature. In the Apollo 2 scanner, the carrier that holds the gel has four integral fluorescent markers for use to correct the image geometry. These markers are a quality control feature that confirms that the scanning has been performed correctly.

In comparison to the scanner described in the Basiji thesis, the optical components of the Apollo 2 scanner have been inverted. In the Apollo 2 scanner, the laser, mirror, waveguide and other optical components are above the glass plate being scanned. The scanner described in the Basiji thesis has these components underneath. In the Apollo 2 scanner, the glass plate is mounted onto the scanner gel side down, so that the optical path remains through the glass plate. By doing this, any particles of gel that may break away from the glass plate will fall onto the base of the instrument rather than into the optics. This does not affect the functionality of the system, but increases its reliability.

Still more preferred is the Apollo 3 scanner, in which the signal output is digitized to the full 16-bit data without any peak saturation or without square root encoding of the signal. A compensation algorithm has also been applied to correct for any variation in detection sensitivity along the path of the scanning beam. This variation is due to anomalies in the optics and differences in collection efficiency across the waveguide. A calibration is performed using a perspex plate with an even fluorescence throughout. The data received from a scan of this plate are used to determine the multiplication factors needed to increase the signal from each pixel level to a target level. These factors are then used in subsequent scans of gels to remove any internal optical variations.

As defined above, a "feature" refers to a spot detected in a 2D gel, and the term "Cardiac Response-Associated Feature" (CRF) refers to a feature that is differentially present in a sample (*e.g.* a sample of serum) from a subject having Cardiac Response compared with a sample (*e.g.* a sample of serum) from a subject free from Cardiac Response.

As used herein, a feature (or a protein isoform of CRPI, as defined *infra*) is "differentially present" in a first sample with respect to a second sample when a method for detecting the feature, isoform or CRPI (*e.g.*, 2D electrophoresis or an immunoassay) gives a different signal when applied to the first and second samples. A feature, isoform or CRPI is "increased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or CRPI is more abundant in the first sample than in the second sample, or if the feature, isoform or CRPI is detectable in the first sample and undetectable in the second sample. Conversely, a feature, isoform or CRPI is "decreased" in the first sample with respect to the second if the method of detection indicates that the feature, isoform or CRPI is less abundant in the first sample than in the second sample or if the feature, isoform or CRPI is undetectable in the first sample and detectable in the second sample.

Preferably, the relative abundance of a feature in two samples is determined in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, *e.g.*, (a) to the total protein in the sample being analyzed (*e.g.*, total protein loaded onto a gel); (b) to an Expression Reference Feature (ERF) *i.e.*, a feature whose abundance is invariant,

within the limits of variability of the Preferred Technology, in the population of subjects being examined, *e.g.* the ERFs disclosed below, or (c) more preferably to the total signal detected from all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

In accordance with an aspect of the present invention, the CRFs disclosed herein have been identified by comparing blood samples from subjects induced to have a Cardiac Response against blood samples from subjects free from Cardiac Response. Subjects free from Cardiac Response include subjects with no known disease or condition (normal subjects). Cardiac response was induced by treatment with doxorubicin (DX) 1mg/kg either alone or 30 min after an i.p. injection of dexrazoxane (ICRF) 50 mg/kg as described in the Examples *infra.* The treatment comprised of 7 weekly injections and samples were taken within 24h after the final injection.

In accordance with another aspect of the invention, another set of CRFs disclosed herein have been identified by comparing blood samples from subjects treated with a cardioprotectant (ICRF) against blood samples from subjects not treated with a cardioprotectant (refer to Examples *infra*).

CRFs have been identified through the methods and apparatus of the Preferred Technology. These CRFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table I.

The CRFs disclosed herein have been identified by comparing the following three sample sets:

1. Blood samples from subjects having cardiac response induced by treatment with DX against blood samples from subjects free from any treatment and cardiac response (DX vs control);

2. Blood samples from subjects having cardiac response induced by treatment with DX in combination with ICRF against blood samples from subjects free from any treatment and cardiac response (DX & ICRF vs control);

3. Blood samples from subjects treated with the cardioprotectant ICRF against blood samples from subjects free from any treatment and cardiac response (ICRF vs control);

Three groups of CRFs have been identified through the methods and apparatus of the Preferred Technology. The first group consists of CRFs that are decreased in the blood of subjects having cardiac response in one or more of the comparisons 1 and 2 described above. These CRFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table I.

Table I. CRFs Decreased in Blood of Subjects Having Cardiac Response

Table I CRF	pI	MW (Da)
CRF-1	5.6	37,430
CRF-2	5.7	12,367
CRF-3	5.3	12,781
CRF-4	5.2	15,589
CRF-5	5.9	67,060
CRF-6	4.6	34,921
CRF-7	5.6	15,634
CRF-8	5.7	99,793
CRF-9	5.2	15,591
CRF-10	5.0	51,899
CRF-11	4.9	52,439
CRF-12	5.0	47,310
CRF-13	6.8	38,368
CRF-14	6.1	39,111
CRF-15	5.7	54,294
CRF-17	6.7	51,594
CRF-18	6.4	76,207
CRF-19	5.7	68,576
CRF-20	5.2	31,123
CRF-21	4.6	36,159
CRF-22	5.7	24,627
CRF-69	5.0	54,619
CRF-72	4.7	39,320

Table I CRF	pI	MW (Da)
CRF-79	6.8	80,678
CRF-80	6.6	81,686
CRF-82	7.6	77,393
CRF-84	4.8	73,524
CRF-87	6.2	67,169
CRF-89	6.1	66,187
CRF-90	5.2	67,336
CRF-92	6.0	63,037
CRF-94	4.7	58,902
CRF-96	4.7	58,255
CRF-98	4.8	57,844
CRF-99	4.9	57,681
CRF-100	4.7	57,646
CRF-102	4.9	56,657
CRF-103	5.0	56,471
CRF-104	7.0	53,989
CRF-105	4.9	52,147
CRF-116	4.4	36,226
CRF-118	6.5	33,117
CRF-121	7.1	27,232
CRF-130	4.8	14,082
CRF-140	5.8	166,485
CRF-141	6.3	107,802
CRF-142	5.4	105,458
CRF-143	6.2	103,573
CRF-144	6.5	57,346
CRF-146	6.4	83,124
CRF-147	6.7	11,959
CRF-148	5.0	62,293
CRF-149	6.6	113,455
CRF-150	4.2	45,541
CRF-152	6.0	53,500
CRF-153	8.0	91,649
CRF-155	4.8	53,058
CRF-156	6.9	78,948
CRF-157	5.2	54,619
CRF-158	8.0	106,079

Table I CRF	pI	MW (Da)
CRF-160	8.1	57,944
CRF-162	9.5	30,585
CRF-165	8.0	51,568
CRF-166	7.9	48,185
CRF-171	8.0	73,106
CRF-173	7.96	78,394
CRF-174	8.0	11,193
CRF-177	4.6	122,135
CRF-178	7.7	77,772
CRF-179	6.4	119,866
CRF-181	4.4	12,568
CRF-183	5.1	68,116
CRF-184	10.2	30,035
CRF-185	4.8	72,982
CRF-186	7.2	50,464

The second group consists of CRFs that are increased in the blood of subjects having cardiac response in one or more of the comparisons 1 and 2 described above. These CRFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table II.

Table II. CRFs Increased in Blood of Subjects Having Cardiac Response

Table II CRF	pI	MW (Da)
CRF-42	4.6	15,989
CRF-43	5.6	41,054
CRF-44	5.8	52,589
CRF-45	5.3	31,522
CRF-47	4.4	11,368
CRF-49	6.2	47,119
CRF-51	4.6	23,573
CRF-52	5.4	22,411
CRF-53	5.6	29,885
CRF-54	5.6	37,507
CRF-58	6.8	34,063

Table II CRF	pI	MW (Da)
CRF-59	5.8	36,833
CRF-62	7.2	33,555
CRF-66	6.6	34,783
CRF-83	5.8	78,180
CRF-95	4.9	59,635
CRF-112	7.1	47,499
CRF-113	6.4	46,986
CRF-115	7.1	38,136
CRF-119	5.8	30,680
CRF-120	4.9	29,904
CRF-122	5.3	24,915
CRF-124	5.0	23,549
CRF-127	4.8	23,325
CRF-128	5.3	21,197
CRF-129	5.4	17,765
CRF-131	4.3	11,917
CRF-132	4.5	10,104
CRF-134	5.1	51,125
CRF-135	7.3	85,176
CRF-136	7.3	95,488
CRF-145	5.7	34335
CRF-151	5.9	48626
CRF-154	8.0	56174
CRF-159	4.4	50130
CRF-161	5.3	187979
CRF-163	4.6	44825
CRF-164	4.5	67788
CRF-167	4.9	38954
CRF-168	4.8	30656
CRF-169	9.3	63133
CRF-170	4.8	34688
CRF-172	4.5	54186
CRF-175	5.6	49354
CRF-176	5.3	80734
CRF-180	4.5	44813
CRF-182	5.1	180157

The third group consists of CRFs that are (a) decreased and (b) increased in the blood of subjects treated with a cardioprotectant in comparison 3 described above. These CRFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as provided in Table III (a) and (b).

Table III. CRFs Altered in Blood of Subjects Treated with a Cardioprotectant.

Table III		
(a) CRF	**pI**	**MW (Da)**
CRF-187	5.1	25,055
CRF-188	9.2	24,865
CRF-189	5.4	57,618
CRF-28	5.7	26,409
(b)		
CRF-73	5.4	29,367
CRF-56	4.9	48,318
CRF-190	4.8	65,376

For any given CRF, the signal obtained upon analyzing blood from subjects having cardiac response relative to the signal obtained upon analyzing blood from subjects free from cardiac response will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the present invention contemplates that each laboratory will, based on the present description, establish a reference range for each CRF in subjects free from cardiac response according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one control positive blood sample from a subject known to have cardiac response or at least one control negative blood sample from a subject known to be free from cardiac response (and more preferably both positive and negative control samples) are included in each batch of test samples analyzed. In one embodiment, the level of expression of a feature is determined relative to a background value, which is defined as the level of signal obtained from a proximal region of the image that (a) is equivalent in area to the particular feature in question; and (b) contains no discernable protein feature.

In a preferred embodiment, the signal associated with a CRF in the blood of a subject (e.g., a subject suspected of having or known to have cardiac response) is normalized with reference to one or more ERFs detected in the same 2D gel. As

will be apparent to one of ordinary skill in the art, such ERFs may readily be determined by comparing different samples using the Preferred Technology. Suitable ERFs include (but are not limited to) that described in the following table.

Table IV. Expression Reference Features in Blood

ERF	pI	MW (Da)
ERF-1	6.9	48,923
ERF-2	7.2	41,803
ERF-3	6.7	33,146
ERF-4	5.4	24,541
ERF-5	4.6	21,845

As those of skill in the art will readily appreciate, the measured MW and pI of a given feature or protein isoform will vary to some extent depending on the precise protocol used for each step of the 2D electrophoresis and for landmark matching. As used herein, the terms "MW" and "pI" are defined, respectively, to mean the apparent molecular weight and the apparent isoelectric point of a feature or protein isoform as measured in exact accordance with the Reference Protocol identified in Section 6 below. When the Reference Protocol is followed and when samples are run in duplicate or a higher number of replicates, variation in the measured mean pI of a CRF or CRPI is typically less than 3% and variation in the measured mean MW of a CRF or CRPI is typically less than 5%. Where the skilled artisan wishes to deviate from the Reference Protocol, calibration experiments should be performed to compare the MW and pI for each CRF or protein isoform as detected (a) by the Reference Protocol and (b) by the deviant protocol.

CRFs can be used for detection, prognosis, diagnosis, or monitoring of cardiac response, or for identifying patients most likely to respond to a specific therapeutic treatment, or for drug development. In one embodiment of the invention, blood from a subject (*e.g.*, a subject treated with a drug candidate, or suspected of having cardiac response) is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CRFs: CRF-1, CRF-2, CRF-3, CRF-4, CRF-5, CRF-6, CRF-7, CRF-8, CRF-9, CRF-10, CRF-11, CRF-12, CRF-13, CRF-14, CRF-15,

CRF-17, CRF-18, CRF-19, CRF-20, CRF-21, CRF-22, CRF-56, CRF-69, CRF-72, CRF-73, CRF-79, CRF-80, CRF-82, CRF-84, CRF-87, CRF-89, CRF-90, CRF-92, CRF-94, CRF-96, CRF-98, CRF-99, CRF-100, CRF-102, CRF-103, CRF-104, CRF-105, CRF-116, CRF-118, CRF-121, CRF-130, CRF-140, CRF-141, CRF-142, CRF-143, CRF-144, CRF-146, CRF-147, CRF-148, CRF-149, CRF-150, CRF-152, CRF-153, CRF-155, CRF-156, CRF-157, CRF-158, CRF-160, CRF-162, CRF-165, CRF-166, CRF-171, CRF-173, CRF-174, CRF-177, CRF-178, CRF-179, CRF-181, CRF-183, CRF-184, CRF-185, CRF-186, CRF-190. A decreased abundance of said one or more CRFs in the blood from the subject relative to blood from a subject or subjects free from cardiac response (*e.g.*, a control sample or a previously determined reference range) indicates the presence of cardiac response.

In another embodiment of the invention, blood from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CRFs: CRF-28, CRF-42, CRF-43, CRF-44, CRF-45, CRF-47, CRF-49, CRF-51, CRF-52, CRF-53, CRF-54, CRF-58, CRF-59, CRF-62, CRF-66, CRF-83, CRF-95, CRF-112, CRF-113, CRF-115, CRF-119, CRF-120, CRF-122, CRF-124, CRF-127, CRF-128, CRF-129, CRF-131, CRF-132, CRF-134, CRF-135, CRF-136, CRF-145, CRF-151, CRF-154, CRF-159, CRF-161, CRF-163, CRF-164, CRF-167, CRF-168, CRF-169, CRF-170, CRF-172, CRF-175, CRF-176, CRF-180, CRF-182, CRF-187, CRF-188, CRF-189. An increased abundance of said one or more CRFs in the blood from the subject relative to blood from a subject or subjects free from cardiac response (*e.g.*, a control sample or a previously determined reference range) indicates the presence of cardiac response.

In yet another embodiment, blood from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CRFs or any combination of them, whose decreased abundance indicates the presence of cardiac response i.e. CRF-1, CRF-2, CRF-3, CRF-4, CRF-5, CRF-6, CRF-7, CRF-8, CRF-9, CRF-10, CRF-11, CRF-12, CRF-13, CRF-14, CRF-15, CRF-17, CRF-18, CRF-19, CRF-20, CRF-21, CRF-22, CRF-56, CRF-69, CRF-72, CRF-73, CRF-79, CRF-80, CRF-82, CRF-84, CRF-87, CRF-89, CRF-90, CRF-92, CRF-94, CRF-96, CRF-98, CRF-99, CRF-100, CRF-102, CRF-103, CRF-104, CRF-105, CRF-116, CRF-118, CRF-121, CRF-130, CRF-140, CRF-141, CRF-142, CRF-143, CRF-144, CRF-146, CRF-147, CRF-148, CRF-149, CRF-150, CRF-152, CRF-153, CRF-155, CRF-156, CRF-157, CRF-158, CRF-160, CRF-162, CRF-165, CRF-166, CRF-171, CRF-173, CRF-174, CRF-177, CRF-178, CRF-179, CRF-181, CRF-183, CRF-184, CRF-185,

CRF-186, CRF-190, and (b) one or more CRFs or any combination of them, whose increased abundance indicates the presence of cardiac response *i.e.* CRF-28, CRF-42, CRF-43, CRF-44, CRF-45, CRF-47, CRF-49, CRF-51, CRF-52, CRF-53, CRF-54, CRF-58, CRF-59, CRF-62, CRF-66, CRF-83, CRF-95, CRF-112, CRF-113, CRF-115, CRF-119, CRF-120, CRF-122, CRF-124, CRF-127, CRF-128, CRF-129, CRF-131, CRF-132, CRF-134, CRF-135, CRF-136, CRF-145, CRF-151, CRF-154, CRF-159, CRF-161, CRF-163, CRF-164, CRF-167, CRF-168, CRF-169, CRF-170, CRF-172, CRF-175, CRF-176, CRF-180, CRF-182, CRF-187, CRF-188, CRF-189. In a preferred embodiment, blood from a subject is analyzed for quantitative detection of a plurality of CRFs.

In yet another embodiment of the invention, blood from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more of the following CRFs: CRF-1, CRF-2, CRF-3, CRF-4, CRF-5, CRF-6, CRF-7, CRF-8, CRF-9, CRF-10, CRF-11, CRF-12, CRF-13, CRF-14, CRF-15, CRF-17, CRF-18, CRF-19, CRF-20, CRF-21, CRF-22, CRF-28, CRF-42, CRF-43, CRF-44, CRF-45, CRF-47, CRF-49, CRF-51, CRF-52, CRF-53, CRF-54, CRF-56, CRF-58, CRF-59, CRF-62, CRF-66, CRF-69, CRF-72, CRF-73, CRF-79, CRF-80, CRF-82, CRF-83, CRF-84, CRF-87, CRF-89, CRF-90, CRF-92, CRF-94, CRF-95, CRF-96, CRF-98, CRF-99, CRF-100, CRF-102, CRF-103, CRF-104, CRF-105, CRF-112, CRF-113, CRF-115, CRF-116, CRF-118, CRF-119, CRF-120, CRF-121, CRF-122, CRF-124, CRF-127, CRF-128, CRF-129, CRF-130, CRF-131, CRF-132, CRF-134, CRF-135, CRF-136, CRF-140, CRF-141, CRF-142, CRF-143, CRF-144, CRF-145, CRF-146, CRF-147, CRF-148, CRF-149, CRF-150, CRF-151, CRF-152, CRF-153, CRF-154, CRF-155, CRF-156, CRF-157, CRF-158, CRF-159, CRF-160, CRF-161, CRF-162, CRF-163, CRF-164, CRF-165, CRF-166, CRF-167, CRF-168, CRF-169, CRF-170, CRF-171, CRF-172, CRF-173, CRF-174, CRF-175, CRF-176, CRF-177, CRF-178, CRF-179, CRF-180, CRF-181, CRF-182, CRF-183, CRF-184, CRF-185, CRF-186, CRF-187, CRF-188, CRF-189, CRF-190 wherein the ratio of the one or more CRFs relative to an Expression Reference Feature (ERF) indicates whether cardiac response is present.

In a specific embodiment, a decrease in one or more CRF/ERF ratios in a test sample relative to the CRF/ERF ratios in a control sample or a reference range indicates the presence of cardiac response; CRF-1, CRF-2, CRF-3, CRF-4, CRF-5, CRF-6, CRF-7, CRF-8, CRF-9, CRF-10, CRF-11, CRF-12, CRF-13, CRF-14, CRF-15, CRF-17, CRF-18, CRF-19, CRF-20, CRF-21, CRF-22, CRF-56, CRF-69, CRF-

72, CRF-73, CRF-79, CRF-80, CRF-82, CRF-84, CRF-87, CRF-89, CRF-90, CRF-92, CRF-94, CRF-96, CRF-98, CRF-99, CRF-100, CRF-102, CRF-103, CRF-104, CRF-105, CRF-116, CRF-118, CRF-121, CRF-130, CRF-140, CRF-141, CRF-142, CRF-143, CRF-144, CRF-146, CRF-147, CRF-148, CRF-149, CRF-150, CRF-152, CRF-153, CRF-155, CRF-156, CRF-157, CRF-158, CRF-160, CRF-162, CRF-165, CRF-166, CRF-171, CRF-173, CRF-174, CRF-177, CRF-178, CRF-179, CRF-181, CRF-183, CRF-184, CRF-185, CRF-186, CRF-190, are suitable CRFs for this purpose. In another specific embodiment, an increase in one or more CRF/ERF ratios in a test sample relative to the CRF/ERF ratios in a control sample or a reference range indicates the presence of cardiac response; CRF-28, CRF-42, CRF-43, CRF-44, CRF-45, CRF-47, CRF-49, CRF-51, CRF-52, CRF-53, CRF-54, CRF-58, CRF-59, CRF-62, CRF-66, CRF-83, CRF-95, CRF-112, CRF-113, CRF-115, CRF-119, CRF-120, CRF-122, CRF-124, CRF-127, CRF-128, CRF-129, CRF-131, CRF-132, CRF-134, CRF-135, CRF-136, CRF-145, CRF-151, CRF-154, CRF-159, CRF-161, CRF-163, CRF-164, CRF-167, CRF-168, CRF-169, CRF-170, CRF-172, CRF-175, CRF-176, CRF-180, CRF-182, CRF-187, CRF-188, CRF-189, are suitable CRFs for this purpose.

In a further embodiment of the invention, blood from a subject is analyzed by 2D electrophoresis for quantitative detection of (a) one or more CRFs, or any combination of them, whose decreased CRF/ERF ratio(s) in a test sample relative to the CRF/ERF ratio(s) in a control sample indicates the presence of cardiac response, *i.e.,* CRF-1, CRF-2, CRF-3, CRF-4, CRF-5, CRF-6, CRF-7, CRF-8, CRF-9, CRF-10, CRF-11, CRF-12, CRF-13, CRF-14, CRF-15, CRF-17, CRF-18, CRF-19, CRF-20, CRF-21, CRF-22, CRF-56, CRF-69, CRF-72, CRF-73, CRF-79, CRF-80, CRF-82, CRF-84, CRF-87, CRF-89, CRF-90, CRF-92, CRF-94, CRF-96, CRF-98, CRF-99, CRF-100, CRF-102, CRF-103, CRF-104, CRF-105, CRF-116, CRF-118, CRF-121, CRF-130, CRF-140, CRF-141, CRF-142, CRF-143, CRF-144, CRF-146, CRF-147, CRF-148, CRF-149, CRF-150, CRF-152, CRF-153, CRF-155, CRF-156, CRF-157, CRF-158, CRF-160, CRF-162, CRF-165, CRF-166, CRF-171, CRF-173, CRF-174, CRF-177, CRF-178, CRF-179, CRF-181, CRF-183, CRF-184, CRF-185, CRF-186, CRF-190, and (b) one or more CRFs, or any combination of them, whose increased CRFF/ERF ratio(s) in a test sample relative to the CRF/ERF ratio(s) in a control sample indicates the presence of cardiac response, *i.e.,* CRF-28, CRF-42, CRF-43, CRF-44, CRF-45, CRF-47, CRF-49, CRF-51, CRF-52, CRF-53, CRF-54, CRF-58, CRF-59, CRF-62, CRF-66, CRF-83, CRF-95, CRF-112, CRF-113, CRF-

115, CRF-119, CRF-120, CRF-122, CRF-124, CRF-127, CRF-128, CRF-129, CRF-131, CRF-132, CRF-134, CRF-135, CRF-136, CRF-145, CRF-151, CRF-154, CRF-159, CRF-161, CRF-163, CRF-164, CRF-167, CRF-168, CRF-169, CRF-170, CRF-172, CRF-175, CRF-176, CRF-180, CRF-182, CRF-187, CRF-188, CRF-189. In a preferred embodiment, blood from a subject is analyzed for quantitative detection of a plurality of CRFs.

5.3 Cardiac Response-Associated Protein Isoforms (CRPIs)

In another aspect of the invention, blood from a subject, preferably a living subject, is analyzed for quantitative detection of one or more Cardiac Response-Associated Protein Isoforms (CRPIs) for screening or diagnosis of cardiac response, to determine the prognosis of a subject having cardiac response, to monitor the effectiveness of cardiac response therapy, for identifying patients most likely to respond to a particular therapeutic treatment or for drug development, and, in particular, to determine the potential for drug candidates to induce a vascular response. As is well known in the art, a given protein may be expressed as one or more variants (isoforms) that differ in their amino acid composition (*e.g.* as a result of alternative mRNA or premRNA processing, *e.g.* alternative splicing or limited proteolysis) or as a result of differential post-translational modification (*e.g.*, glycosylation, phosphorylation, acylation), or both. Thus, proteins of identical amino acid sequence or proteins encoded by a single gene can differ in their pI, MW, or both. It follows that differential presence of a protein isoform does not require differential expression of the gene encoding the protein in question. As used herein, the term "Cardiac Response-Associated Protein Isoform" refers to a protein isoform that is differentially present in a sample of blood from a subject having cardiac response compared with sample of blood from a subject free from cardiac response. As used herein, the term "isoform" also refers to a protein that exists in only a single form, *i.e.,* it is not expressed as several variants.

Three groups of CRPIs have been identified by amino acid sequencing of CRFs. CRPIs were isolated, subjected to proteolysis, and analyzed by mass spectrometry using the methods and apparatus of the Preferred Technology. One skilled in the art can identify sequence information from proteins analyzed by mass spectrometry and/or tandem mass spectrometry using various spectral interpretation methods and database searching tools. Examples of some of these methods and tools can be found at the Swiss Institute of Bioinformatics web site at

http://www.expasy.com/, and the European Molecular Biology Laboratory web site at www.mann.embl-heidelberg.de/Services/PeptideSearch/. Identification of CRPIs was performed primarily using the SEQUEST search program (Eng et al., 1994, J. Am. Soc. Mass Spectrom. 5:976-989) with raw, uninterpreted tandem mass spectra of tryptic digest peptides as described in the Examples, *infra.*

The first group consists of CRPIs that are decreased in the blood of subjects having cardiac response as described in comparisons 1 and 2 (section 5.2). The amino acid sequences of tryptic digest peptides of these CRPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed in Table V in addition to the pIs and MWs of these CRPIs.

Table V. CRPIs Decreased in Blood of Subjects Having Cardiac Response

Table V CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
CRF-1	CRPI-1	5.6	37,430	TADGSWEPFASGK	SEQ ID 55
				TAESGELHGLTTDEK	SEQ ID 56
CRF-2	CRPI-2	5.7	12,367	TADGSWEPFASGK	SEQ ID 55
				TAESGELHGLTTDEK	SEQ ID 56
CRF-4	CRPI-4	5.2	15,589	ELYLVAYK	SEQ ID 19
				NGETFQAMVLYGR	SEQ ID 44
CRF-5	CRPI-5	5.9	67,060	CNADPGLSALLSDHR	SEQ ID 9
				DYFISCPGR	SEQ ID 17
				FNPVTGEVPPR	SEQ ID 25
				NPVTSVDAAFR	SEQ ID 45
				GECQSEGVLFFQGNR	SEQ ID 26
				SGAQATWAELSWPHEK	SEQ ID 52
				VWVYPPEK	SEQ ID 66
CRF-7	CRPI-7	5.6	15,634	DNEEFLESNK	SEQ ID 15
CRF-8	CRPI-8	5.7	99,793	EPGLQIWR	SEQ ID 20
				YIETDPANR	SEQ ID 70
CRF-10	CRPI-10	5.0	51,899	MQHLEQTLTK	SEQ ID 43
				RPFNPEHTR	SEQ ID 50
				VFNNDADLSGITEDAPLK	SEQ ID 62
CRF-11	CRPI-11	4.9	52,439	AVLTLDER	SEQ ID 7
				DADFHVDK	SEQ ID 10
				MQHLEQTLTK	SEQ ID 43

Table V CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
				RPFNPEHTR	SEQ ID 50
				VFNNDADLSGITEDAPLK	SEQ ID 62
				VINDYVEK	SEQ ID 63
CRF-14	CRPI-14	6.1	39,111	TADGSWEPFASGK	SEQ ID 55
				TAESGELHGLTTDEK	SEQ ID 56
CRF-15	CRPI-15	5.7	54,294	AMFHVNKPR	SEQ ID 2
				GSIQHLPEQEEPEDSK	SEQ ID 29
				DGYMLTLNR	SEQ ID 13
				KLVVLPFPGK	SEQ ID 35
CRF-19	CRPI-19	5.7	68,576	CNADPGLSALLSDHR	SEQ ID 9
				DYFISCPGR	SEQ ID 17
				FNPVTGEVPPR	SEQ ID 25
				GECQSEGVLFFQGNR	SEQ ID 26
				NPVTSVDAAFR	SEQ ID 45
				SGAQATWAELSWPHEK	SEQ ID 52
				VWVYPPEK	SEQ ID 66
CRF-69	CRPI-69	5.2	54,619	QLSLLTTMSNR	SEQ ID 48
				RTQVPEVFLSK	SEQ ID 51
				VCSQYAAYGK	SEQ ID 61
				YCSSQIDAEMR	SEQ ID 69
CRF-92	CRPI-92.1	6.0	63,037	FNPVTGEVPPR	SEQ ID 25
				VWVYPPEK	SEQ ID 66
				NPVTSVDAAFR	SEQ ID 45
CRF-92	CRPI-92.2	6.0	63,037	LGEYGFQNAVLVR	SEQ ID 38
				SIHTLFGDK	SEQ ID 53
				APQVSTPTLVEAAR	SEQ ID 3
CRF-100	CRPI-100	4.7	57,646	IAELFSELDER	SEQ ID 31
				DSTMEEILEGLK	SEQ ID 16
				KIFSQQADLSR	SEQ ID 33
				DLTTPYIR	SEQ ID 14
				EQPILSEFQEK	SEQ ID 21
				GFGHLLQR	SEQ ID 27
CRF-105	CRPI-105	4.9	52,147	VFNNDADLSGITEDAPLK	SEQ ID 62
				AVLTLDER	SEQ ID 7
CRF-140	CRPI-140	5.8	166,485	AISYLISGYQR	SEQ ID 1
CRF-150	CRPI-150	4.2	45,541	KPDLSPELR	SEQ ID 36

Table V CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
				QQLELEK	SEQ ID 49
CRF-156	CRPI-156	6.9	78,948	IPSHAVVAR	SEQ ID 32
				GTDFQLNQLQGK	SEQ ID 30
				ASDSSINWNNLK	SEQ ID 5
				LYLGHSYVTAIR	SEQ ID 41

The second group consists of CRPIs that are increased in the blood of subjects having cardiac response as described in comparisons 1 and 2 (section 5.2). The amino acid sequences of tryptic digest peptides of these CRPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed in Table VI in addition to the pIs and MWs of these CRPIs.

Table VI. CRPIs Increased in Blood of Subjects Having Cardiac Response

Table VI CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
CRF-43	CRPI-43	5.6	41,054	TVGVWSPSPPTCER	SEQ ID 60
				VLHGTINSGFK	SEQ ID 64
CRF-44	CRPI-44	5.8	52,589	NTAPTSSPSLTAPR	SEQ ID 46
				DGYMLTLNR	SEQ ID 13
				LFYETVHGQCK	SEQ ID 37
				NTAPTSSPSLTAPR	SEQ ID 46
CRF-51	CRPI-51	4.6	23,573	WNEEVEAYR	SEQ ID 68
CRF-52	CRPI-52	5.4	22,411	FGLYSDQMR	SEQ ID 24
				WNEEVEAYR	SEQ ID 67
CRF-54	CRPI-54	5.6	37,507	LQDQSNIQR	SEQ ID 40
				TEVNTNHVLIYIEK	SEQ ID 57
				VNTLPLNFDK	SEQ ID 65
CRF-59	CRPI-59	5.8	36,833	KLQDQSNIQR	SEQ ID 34
				LQDQSNIQR	SEQ ID 40
CRF-95	CRPI-95	4.9	59,635	FATNFYQHLADSK	SEQ ID 23
				EVALNTIIFMGR	SEQ ID 22
CRF-122	CRPI-122	5.3	24,915	WNEEVEAYR	SEQ ID 68
CRF-124	CRPI-124	5.0	23,549	DFATVYVDAVK	SEQ ID 11
				FGLYSDQMR	SEQ ID 24

Table VI CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
				WNEEVEAYR	SEQ ID 67
CRF-132	CRPI-132	4.5	10,104	TGTNLMDFLSR	SEQ ID 58
				AQPSEIQNQAK	SEQ ID 4
				AYFQNAQER	SEQ ID 8
CRF-134	CRPI-134	5.1	51,125	TSTADYAMFR	SEQ ID 59
CRF-154	CRPI-154	8.0	56,174	GLIDEANQDFTNR	SEQ ID 28
				MHPELGSFYDSR	SEQ ID 42
				SQLQEGPPEWK	SEQ ID 54
CRF-164	CRPI-164	4.5	67,788	DGAETLYSFK	SEQ ID 12
				EDFPFLR	SEQ ID 18
				YVIEFIAR	SEQ ID 72
				YNAELESGNQFLLYR	SEQ ID 71

The third group consists of CRPIs that are increased in the blood of subjects treated with a cardioprotectant as described in comparison 3 (section 5.2). The amino acid sequences of tryptic digest peptides of these CRPIs identified by tandem mass spectrometry and database searching as described in the Examples, *infra* are listed in Table VII in addition to the pIs and MWs of these CRPIs.

Table VII CRPIs Increased in Blood of Subjetcs Treated with a Cardioprotectant

Table VII CRF	CRPI	pI	MW (Da)	Amino Acid Sequences of Tryptic Digest Peptides	Sequence Listing SEQ ID
CRF-56	CRPI-56	4.9	48,318	ATIDQNLEDLR	SEQ ID 6
				LGNINTYADDLQNK	SEQ ID 39
				QLDQQVEVFR	SEQ ID 47

As will be evident to one of skill in the art, based upon the present description, a given CRPI can be described according to the data provided for that CRPI in Table V, VI or VII. The CRPI is a protein comprising a peptide sequence described for that CRPI (preferably comprising a plurality of, more preferably all of,

the peptide sequences described for that CRPI) and has a pI of about the value stated for that CRPI (preferably within 10%, more preferably within 5% still more preferably within 1% of the stated value) and has a MW of about the value stated for that CRPI (preferably within 10%, more preferably within 5%, still more preferably within 1% of the stated value).

In one embodiment, blood from a subject is analyzed for quantitative detection of one or more of the following CRPIs: CRPI-1, CRPI-2, CRPI-4, CRPI-5, CRPI-7, CRPI-8, CRPI-10, CRPI-11, CRPI-14, CRPI-15, CRPI-19, CRPI-69, CRPI-92.1, CRPI-92.2, CRPI-100, CRPI-105, CRPI-140, CRPI-150, CRPI-156, CRPI-56 or any combination of them, wherein a decreased abundance of the CRPI or CRPIs (or any combination of them) in the blood from the subject relative to blood from a subject or subjects free from cardiac response (*e.g.*, a control sample or a previously determined reference range) indicates the presence of cardiac response.

In another embodiment of the invention, blood from a subject is analyzed for quantitative detection of one or more of the following CRPIs: CRPI-43, CRPI-44, CRPI-51, CRPI-52, CRPI-54, CRPI-59, CRPI-95, CRPI-122, CRPI-124, CRPI-132, CRPI-134, CRPI-154, CRPI-164 or any combination of them, wherein an increased abundance of the CRPI or CRPIs (or any combination of them) in blood from the subject relative to blood from a subject or subjects free from cardiac response (*e.g.*, a control sample or a previously determined reference range) indicates the presence of cardiac response.

In a further embodiment, blood from a subject is analyzed for quantitative detection of (a) one or more CRPIs, or any combination of them, whose decreased abundance indicates the presence of cardiac response, *i.e.,* CRPI-1, CRPI-2, CRPI-4, CRPI-5, CRPI-7, CRPI-8, CRPI-10, CRPI-11, CRPI-14, CRPI-15, CRPI-19, CRPI-69, CRPI-92.1, CRPI-92.2, CRPI-100, CRPI-105, CRPI-140, CRPI-150, CRPI-156, CRPI-56and (b) one or more CRPIs, or any combination of them, whose increased abundance indicates the presence of cardiac response, *i.e.* CRPI-43, CRPI-44, CRPI-51, CRPI-52, CRPI-54, CRPI-59, CRPI-95, CRPI-122, CRPI-124, CRPI-132, CRPI-134, CRPI-154, CRPI-164. In yet a further embodiment, blood from a subject is analyzed for quantitative detection of one or more CRPIs and one or more previously known biomarkers of cardiac response (*e.g.*, histology, soft tissue imaging). In accordance with this embodiment, the abundance of each CRPI and known biomarker relative to a control or reference range indicates whether a subject has cardiac response.

Preferably, the abundance of a CRPI is normalized to an Expression Reference Protein Isoform (ERPI). ERPIs can be identified by partial amino acid sequencing of ERFs, which are described above, using the methods and apparatus of the Preferred Technology.

As shown above, the CRPIs described herein include previously unknown proteins, as well as isoforms of known proteins where the isoforms were not previously known to be associated with cardiac response. For each CRPI, the present invention additionally provides: (a) a preparation comprising the isolated CRPI; (b) a preparation comprising one or more fragments of the CRPI; and (c) antibodies that bind to said CRPI, to said fragments, or both to said CRPI and to said fragments. As used herein, a CRPI is "isolated" when it is present in a preparation that is substantially free of contaminating proteins, *i.e.*, a preparation in which less than 10% (preferably less than 5%, more preferably less than 1%) of the total protein present is contaminating protein(s). A contaminating protein is a protein having a significantly different pI or MW from those of the isolated CRPI, as determined by 2D electrophoresis. As used herein, a "significantly different" pI or MW is one that permits the contaminating protein to be resolved from the CRPI on 2D electrophoresis, performed according to the Reference Protocol.

In one embodiment, an isolated protein is provided, said protein comprising a peptide with the amino acid sequence identified in Table V, VI or VII for a CRPI, said protein having a pI and MW within 10% (preferably within 5%, more preferably within 1%) of the values identified in Table V, VI or VII for that CRPI.

The CRPIs of the invention can be qualitatively or quantitatively detected by any method known to those skilled in the art, including but not limited to the Preferred Technology described herein, kinase assays, enzyme assays, binding assays and other functional assays, immunoassays, and western blotting. In one embodiment, the CRPIs are separated on a 2-D gel by virtue of their MWs and pIs and visualized by staining the gel. In one embodiment, the CRPIs are stained with a fluorescent dye and imaged with a fluorescence scanner. Sypro Red (Molecular Probes, Inc., Eugene, Oregon) is a suitable dye for this purpose. A preferred fluorescent dye is Pyridinium, 4-[2-[4-(dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt. *See* U.S. Application No. 09/412,168, filed on October 5, 1999, which is incorporated herein by reference in its entirety.

Alternatively, CRPIs can be detected in an immunoassay. In one embodiment, an immunoassay is performed by contacting a sample from a subject to

be tested with an anti-CRPI antibody under conditions such that immunospecific binding can occur if the CRPI is present, and detecting or measuring the amount of any immunospecific binding by the antibody. Anti-CRPI antibodies can be produced by the methods and techniques taught herein; examples of such antibodies known in the art which have been reported to recognize a protein having the amino acid sequence corresponding to a sequence of a CRPI, or which have been reported to recognize a protein named in the database selected by searching with the CRPI sequence are set forth in Table VIII. These antibodies shown in Table VIII are already reported to bind to the protein of which the CRPI is itself predicted to be a family member.

Table VIII Known Antibodies That May Recognise CRPIs or CRPI-Related Polypeptides

CRF	CRPI	Antibody	Manufacturer	Catalogue Number
CRF-92	CRPI-92.2	Albumin, Human, Chicken anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	IMS- 01-026-02
CRF-95	CRPI-95	Antithrombin III, Clone: BL-ATIII/3, Mab anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	BYA- 9009-1
CRF-51, CRF-52, CRF-122, CRF-124	CRPI-51, CRPI-52, CRPI-122, CRPI-124	Apolipoprotein A1 (HDL), Sheep anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	ACL- 20075AP
CRF-56	CRPI-56	Apolipoprotein A1 (HDL), Sheep anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	ACL- 20075AP
CRF-8	CRPI-8	Gelsolin, plasma + cytoplasmic, Sheep anti-	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YBG- 4628-6210
CRF-5, CRF-19, CRF-92	CRPI-5, CRPI-19, CRPI-92.1	Hemopexin, Beta-1, Rabbit anti-Human, precipitating	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	YN- RHHPX
CRF-54, CRF-59	CRPI-54, CRPI-59	rabbit anti-human alpha-2-macroglobulin	Cambio Ltd.	CA-0427

CRF	CRPI	Antibody	Manufacturer	Catalogue Number
CRF-150	CRPI-150	Rabbit Polyclonal Anti-Human alpha-1-acid-Glycoprotein	Biogenesis Ltd.	4729-9957
CRF-7	CRPI-7	Rabbit Polyclonal Anti-Human Ceruloplasmin	DAKO CORPORATION	A0031
CRF-1, CRF-2, CRF-14	CRPI-1, CRPI-2, CRPI-14	Transthyretin, Prealbumin, 55kD, Rabbit anti-Human	ACCURATE CHEMICAL & SCIENTIFIC CORPORATION	MED- CLA 193

Particularly, the anti-CRPI antibody preferentially binds to the CRPI rather than to other isoforms of the same protein. In a particular embodiment, the anti-CRPI antibody binds to the CRPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms of the same protein. The skilled artisan can readily assess and determine the ability of the noted antibody to recognize or bind to the CRPI and the specificity of such binding or recognition. When the antibodies shown in Table VIII do not display the required preferential selectivity for the target CRPI, one skilled in the art can generate additional antibodies by using the CRPI itself for the generation of such antibodies.

CRPIs can be transferred from the gel to a suitable membrane (*e.g.* a PVDF membrane) and subsequently probed in suitable assays that include, without limitation, competitive and non-competitive assay systems using techniques such as western blots and "sandwich" immunoassays using anti-CRPI antibodies as described herein, e.g. the antibodies identified in Table VIII, or others raised against the CRPIs of interest as those skilled in the art will appreciate based on the present description. The immunoblots can be used to identify those anti-CRPI antibodies displaying the selectivity required to immuno-specifically differentiate a CRPI from other isoforms encoded by the same gene. For example, one skilled in the art can identify anti-CRPI antibodies in catalogues of commercially available antibodies. Some examples of companies that supply antibodies include Accurate Chemical & Scientific Corporation (www.accuratechemical.com), Cambio Ltd. (www.cambio.com), Biogenesis Ltd. (www.biodesign.com), and Dako Corporation (www.dako.com).

In one embodiment, binding of antibody in tissue sections can be used to detect aberrant CRPI localization or an aberrant level of one or more CRPIs. In a specific embodiment, antibody to a CRPI can be used to assay a tissue sample (*e.g.*, a cardiac biopsy) from a subject for the level of the CRPI where an aberrant level of CRPI is indicative of cardiac response. As used herein, an "aberrant level" means a level that is increased or decreased compared with the level in a subject free from cardiac response or a reference level. If desired, the comparison can be performed with a matched sample from the same subject, taken from a portion of the body not affected by cardiac response.

Any suitable immunoassay can be used, including, without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays.

For example, a CRPI can be detected in a fluid sample (*e.g.*, spinal fluid, blood, plasma, urine, or tissue homogenate) by means of a two-step sandwich assay. In the first step, a capture reagent (e.g., an anti-CRPI antibody) is used to capture the CRPI. Examples of such antibodies known in the art can be identified as described *infra*. The capture reagent can optionally be immobilized on a solid phase. In the second step, a directly or indirectly labeled detection reagent is used to detect the captured CRPI. In one embodiment, the detection reagent is a lectin. Any lectin can be used for this purpose that preferentially binds to the CRPI rather than to other isoforms that have the same core protein as the CRPI or to other proteins that share the antigenic determinant recognized by the antibody. In a preferred embodiment, the chosen lectin binds to the CRPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms that have the same core protein as the CRPI or to said other proteins that share the antigenic determinant recognized by the antibody. Based on the present description, a lectin that is suitable for detecting a given CRPI can readily be identified by methods well known in the art, for instance upon testing one or more lectins enumerated in Table I on pages 158-159 of Sumar et al., Lectins as Indicators of Disease-Associated Glycoforms, *In*: Gabius H-J & Gabius S (eds.), 1993, Lectins and Glycobiology, at pp. 158-174 (which is incorporated herein by

reference in its entirety). Lectins with the desired oligosaccharide specificity can be identified, for example, by their ability to detect the CRPI in a 2D gel, in a replica of a 2D gel following transfer to a suitable solid substrate such as a nitrocellulose membrane, or in a two-step assay following capture by an antibody. In an alternative embodiment, the detection reagent is an antibody, e.g., an antibody that immunospecifically detects other post-translational modifications, such as an antibody that immunospecifically binds to phosphorylated amino acids. Examples of such antibodies include those that bind to phosphotyrosine (BD Transduction Laboratories, catalog nos.: P11230-050/P11230-150; P11120; P38820; P39020), those that bind to phosphoserine (Zymed Laboratories Inc., South San Francisco, CA, catalog no. 61-8100) and those that bind to phosphothreonine (Zymed Laboratories Inc., South San Francisco, CA, catalog nos. 71-8200, 13-9200).

If desired, a gene encoding a CRPI, a related gene, or related nucleic acid sequences or subsequences, including complementary sequences, can also be used in hybridization assays. A nucleotide encoding a CRPI, or subsequences thereof comprising at least 8 nucleotides, preferably at least 12 nucleotides, and most preferably at least 15 nucleotides can be used as a hybridization probe. Hybridization assays can be used for detection, prognosis, diagnosis, or monitoring of conditions, disorders, or disease states, associated with aberrant expression of genes encoding CRPIs, or for differential diagnosis of subjects with signs or symptoms suggestive of cardiac response. In particular, such a hybridization assay can be carried out by a method comprising contacting a subject's sample containing nucleic acid with a nucleic acid probe capable of hybridizing to a DNA or RNA that encodes a CRPI, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization. Nucleotides can be used for therapy of subjects having cardiac response, as described below.

The invention also provides diagnostic kits, comprising an anti-CRPI antibody. In addition, such a kit may optionally comprise one or more of the following: (1) instructions for using the anti-CRPI antibody for diagnosis, prognosis, therapeutic monitoring or any combination of these applications; (2) a labeled binding partner to the antibody; (3) a solid phase (such as a reagent strip) upon which the anti-CRPI antibody is immobilized; and (4) a label or insert indicating regulatory approval for diagnostic, prognostic or therapeutic use or any combination thereof. If no labeled binding partner to the antibody is provided, the anti-CRPI

antibody itself can be labeled with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

The invention also provides a kit comprising a nucleic acid probe capable of hybridizing to RNA encoding a CRPI. In a specific embodiment, a kit comprises in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferably 10-30 nucleotides and still more preferably 10-20 nucleotides) that under appropriate reaction conditions can prime amplification of at least a portion of a nucleic acid encoding a CRPI, such as by polymerase chain reaction (*see, e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Q replicase, cyclic probe reaction, or other methods known in the art.

Kits are also provided which allow for the detection of a plurality of CRPIs or a plurality of nucleic acids each encoding a CRPI. A kit can optionally further comprise a predetermined amount of an isolated CRPI protein or a nucleic acid encoding a CRPI, *e.g.*, for use as a standard or control.

5.3 Statistical Techniques for Identifying CRPIs and CRPI Clusters

The uni-variate differential analysis tools, such as fold changes, wilcoxon rank sum test and t-test, are useful in identifying individual CRFs or CRPIs that are diagnostically associated with cardiac response or in identifying individual CRPIs that regulate the disease process. In most cases, however, those skilled in the art appreciate that the disease process is associated with a combination of CRFs or CRPIs (and to be regulated by a combination of CRPIs), rather than individual CRFs and CRPIs in isolation. The strategies for discovering such combinations of CRFs and CRPIs differ from those for discovering individual CRFs and CRPIs. In such cases, each individual CRF and CRPI can be regarded as one variable and the disease can be regarded as a joint, multi-variate effect caused by interaction of these variables.

The following steps can be used to identify markers from data produced by the Preferred Technology.

The first step is to identify a collection of CRFs or CRPIs that individually show significant association with cardiac response. The association between the identified CRFs or CRPIs and cardiac response need not be as highly significant as is desirable when an individual CRF or CRPI is used as a diagnostic. Any of the tests

discussed above (fold changes, wilcoxon rank sum test, etc.) can be used at this stage. Once a suitable collection of CRFs or CRPIs has been identified, a sophisticated multi-variate analysis capable of identifying clusters can then be used to estimate the significant multivariate associations with cardiac response.

Linear Discriminant Analysis (LDA) is one such procedure, which can be used to detect significant association between a cluster of variables (*i.e.*, CRFs or CRPIs) and cardiac response. In performing LDA, a set of weights is associated with each variable (*i.e.*, CRF or CRPI) so that the linear combination of weights and the measured values of the variables can identify the disease state by discriminating between subjects having cardiac response and subjects free from cardiac response. Enhancements to the LDA allow stepwise inclusion (or removal) of variables to optimize the discriminant power of the model. The result of the LDA is therefore a cluster of CRFs or CRPIs which can be used, without limitation, for diagnosis, prognosis, therapy or drug development. Other enhanced variations of LDA, such as Flexible Discriminant Analysis permit the use of non-linear combinations of variables to discriminate a disease state from a normal state. The results of the discriminant analysis can be verified by post-hoc tests and also by repeating the analysis using alternative techniques such as classification trees.

A further category of CRFs or CRPIs can be identified by qualitative measures by comparing the percentage feature presence of a CRF or CRPI of one group of samples (e.g., samples from diseased subjects) with the percentage feature presence of a CRF or CRPI in another group of samples (e.g., samples from control subjects). The "percentage feature presence" of a CRF or CRPI is the percentage of samples in a group of samples in which the CRF or CRPI is detectable by the detection method of choice. For example, if a CRF is detectable in 95 percent of samples from diseased subjects, the percentage feature presence of that CRF in that sample group is 95 percent. If only 5 percent of samples from non-diseased subjects have detectable levels of the same CRF, detection of that CRF in the sample of a subject would suggest that it is likely that the subject suffers from cardiac response.

5.4 Use in Clinical Studies

The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, *e.g.* to evaluate drugs for therapy of cardiac response. In one embodiment, candidate molecules are tested for their ability to restore CRF or CRPI levels in a subject having cardiac response to levels found in subjects free

from cardiac response or, in a treated subject (*e.g.* after treatment with a cardiotoxic agent), to preserve CRF or CRPI levels at or near non-cardiac response values. The levels of one or more CRFs or CRPIs can be assayed.

In another embodiment, the methods and compositions of the present invention are used to screen candidates for a clinical study to identify individuals having cardiac response; such individuals can then be either excluded from or included in the study or can be placed in a separate cohort for treatment or analysis. If desired, the candidates can concurrently be screened to identify individuals with other measures of cardiac response (*e.g.* echocardiography, troponin level assessment); procedures for these screens are well known in the art.

5.5 Purification of CRPIs

In particular aspects, the invention provides isolated mammalian CRPIs, preferably rat or human CRPIs, and fragments thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" as used herein refers to material displaying one or more functional activities associated with a full-length (wild-type) CRPI, *e.g.*, binding to a CRPI substrate or CRPI binding partner, antigenicity (binding to an anti-CRPI antibody), immunogenicity, enzymatic activity and the like.

In specific embodiments, the invention provides fragments of a CRPI comprising at least 5 amino acids, at least 10 amino acids, at least 50 amino acids, or at least 75 amino acids. Fragments lacking some or all of the regions of a CRPI are also provided, as are proteins (*e.g.*, fusion proteins) comprising such fragments. Nucleic acids encoding the foregoing are provided.

Once a recombinant nucleic acid which encodes the CRPI, a portion of the CRPI, or a precursor of the CRPI is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

The CRPIs identified herein can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once a recombinant nucleic acid that encodes the CRPI is identified, the entire amino acid sequence of the CRPI can be deduced from the nucleotide sequence of the gene coding region contained in the recombinant nucleic acid. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al., 1984, Nature 310:105-111).

In another alternative embodiment, native CRPIs can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).

In a preferred embodiment, CRPIs are isolated by the Preferred Technology described *supra*. For preparative-scale runs, a narrow-range "zoom gel" having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated CRPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated CRPI in a single run. Those of skill in the art will appreciate that a zoom gel can be used in any separation strategy which employs gel isoelectric focusing.

The invention thus provides an isolated CRPI, an isolated CRPI-related polypeptide, and an isolated derivative or fragment of a CRPI or a CRPI-related polypeptide; any of the foregoing can be produced by recombinant DNA techniques or by chemical synthetic methods.

5.6 Isolation of DNA Encoding a CRPI

Specific embodiments for the cloning of a gene encoding a CRPI, are presented below by way of example and not of limitation.

The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding a CRPI or a fragment thereof, or a CRPI-related polypeptide, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification. The nucleotide sequences of the present invention also permit the identification and cloning of the gene encoding a CRPI homolog or CRPI ortholog

including, for example, by screening cDNA libraries, genomic libraries or expression libraries.

For example, to clone a gene encoding a CRPI by PCR techniques, anchored degenerate oligonucleotides (or a set of most likely oligonucleotides) can be designed for all CRPI peptide fragments identified as part of the same protein. PCR reactions under a variety of conditions can be performed with relevant cDNA and genomic DNAs (*e.g.*, from cardiac tissue or from cells of the immune system) from one or more species. Also vectorette reactions can be performed on any available cDNA and genomic DNA using the oligonucleotides (which preferably are nested) as above. Vectorette PCR is a method that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known. Thus, it extends the application of PCR to stretches of DNA where the sequence information is only available at one end. (Arnold C, 1991, PCR Methods Appl. 1(1):39-42; Dyer KD, Biotechniques, 1995, 19(4):550-2). Vectorette PCR may pe performed with probes that are, for example, anchored degenerate oligonucleotides (or most likely oligonucleotides) coding for CRPI peptide fragments, using as a template a genomic library or cDNA library pools.

Anchored degenerate oligonucleotides (and most likely oligonucleotides) can be designed for all CRPI peptide fragments. These oligonucleotides may be labelled and hybridized to filters containing cDNA and genomic DNA libraries. Oligonucleotides to different peptides from the same protein will often identify the same members of the library. The cDNA and genomic DNA libraries may be obtained from any suitable or desired mammalian species, for example from humans.

Nucleotide sequences comprising a nucleotide sequence encoding a CRPI or CRPI fragment of the present invention are useful for their ability to hybridize selectively with complementary stretches of genes encoding other proteins. Depending on the application, a variety of hybridization conditions may be employed to obtain nucleotide sequences at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identical, or 100% identical, to the sequence of a nucleotide encoding a CRPI.

For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, "highly stringent conditions" means hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in

0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent conditions for duplex formation are required. As used herein "moderately stringent conditions" means washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% identical to the fragment of a gene encoding a CRPI, 37°C for 90 to 95% identity and 32°C for 70 to 90% identity.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode parts or the whole of a CRPI. Any suitable method for preparing DNA fragments may be used in the present invention. For example, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T_4, and yeast artificial chromosome (YAC). (*See, e.g.*, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II; Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The genomic library may be screened by nucleic acid hybridization to labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961).

Based on the present description, the genomic libraries may be screened with labeled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the CRPI using optimal approaches well known in the art. Any probe used is at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides,

at least 25 nucleotides, at least 30 nucleotides, at least 40 nucleotides, at least 50 nucleotides, at least 60 nucleotides, at least 70 nucleotides, at least 80 nucleotides, or at least 100 nucleotides. Preferably a probe is 10 nucleotides or longer, and more preferably 15 nucleotides or longer.

In Tables V, VI and VII above, some CRPIs disclosed herein were found to correspond to isoforms of previously identified proteins encoded by genes whose sequences are publicly known. (Sequence analysis and protein identification of CRPIs was carried out using the methods described in Section 6.1.14). To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The SWISS-PROT and trEMBL databases (held by the Swiss Institute of Bioinformatics (SIB) and the European Bioinformatics Institue (EBI) which are available at http://www.expasy.com/) and the GenBank database (held by the National Institute of Health (NIH) which is available at http://www.ncbi.nlm.nih.gov/GenBank/) provide protein sequences comprising the amino acid sequences listed for the CRPIs in Tables V, VI, and VII under the following accession numbers and each sequence is incorporated herein by reference. In many cases the protein sequences in the database will cross-reference a nucleic acid or gene sequence encoding the protein or related protein.

Table IX Sequences encoding CRPIs or CRPI Related Proteins

Table IX CRF	CRPI	Rat/ Mouse* Accession Number	Human Homologue Accession Number
CRF-1	CRPI-1	P02767	P02766
CRF-2	CRPI-2	P02767	P02766
CRF-4	CRPI-4	Q63024	-
CRF-5	CRPI-5	P20059	P02790
CRF-7	CRPI-7	P13635	P00450
CRF-8	CRPI-8	P13020*	P06396
CRF-10	CRPI-10	112889	P01009
CRF-11	CRPI-11	P17475	P01010
CRF-14	CRPI-14	P02767	P02766
CRF-15	CRPI-15	Q9QX79	Q9UGM5

Table IX CRF	CRPI	Rat/ Mouse* Accession Number	Human Homologue Accession Number
CRF-19	CRPI-19	P20059	P02790
CRF-43	CRPI-43	Q63514	P04003
CRF-44	CRPI-44	Q9QX79	Q9UGM5
CRF-51	CRPI-51	P04639	P02647
CRF-52	CRPI-52	P04639	P02647
CRF-54	CRPI-54	Q63332	P01023
CRF-56	CRPI-56	P02651	P06727
CRF-59	CRPI-59	Q63332	P01023
CRF-69	CRPI-69	203941	15295970
CRF-92	CRPI-92.1	P20059	P02790
CRF-92	CRPI-92.2	P02770	P02768
CRF-95	CRPI-95	P32261*	P01008
CRF-100	CRPI-100	207044	-
CRF-105	CRPI-105	P17475	P01009
CRF-122	CRPI-122	P04639	P02647
CRF-124	CRPI-124	P04639	P02647
CRF-132	CRPI-132	P04638	P02652
CRF-134	CRPI-134	P02680	P04469, P02679
CRF-140	CRPI-140	285190	-
CRF-150	CRPI-150	P02764	P02763
CRF-154	CRPI-154	P06399	13591823
CRF-156	CRPI-156	P12346	P02787
CRF-164	CRPI-164	92471	-

For any CRPI, degenerate probes, or probes taken from the sequences described above by accession number may be used for screening. In the case of degenerate probes, they can be constructed from the partial amino sequence information obtained from tandem mass spectra of tryptic digest peptides of the CRPI. To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. When a library is screened, clones with insert DNA encoding the CRPI or a fragment thereof will hybridize to one or more

members of the corresponding set of degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries is carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50_C and washed using the washing conditions described *supra* for highly stringent or moderately stringent hybridization.

In yet another aspect of the invention, clones containing nucleotide sequences encoding the entire CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide any of the foregoing may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed CRPI or CRPI-related polypeptides. In one embodiment, the various anti-CRPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Colonies or plaques from the library are brought into contact with the antibodies to identify those clones that bind antibody.

In an embodiment, colonies or plaques containing DNA that encodes a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide can be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-CRPI antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads are then contacted with colonies or plaques expressing recombinant polypeptides. Colonies or plaques expressing a CRPI or CRPI-related polypeptide are identified as any of those that bind the beads.

Alternatively, the anti-CRPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite® resin. This material is then used to adsorb to bacterial colonies expressing the CRPI protein or CRPI-related polypeptide as described herein.

In another aspect, PCR amplification may be used to isolate from genomic DNA a substantially pure DNA (*i.e.*, a DNA substantially free of contaminating nucleic acids) encoding the entire CRPI or a part thereof. Preferably such a DNA is at least 95% pure, more preferably at least 99% pure. Oligonucleotide sequences, degenerate or otherwise, that correspond to peptide sequences of CRPIs disclosed herein can be used as primers.

PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp® or AmpliTaq DNA polymerase). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding a CRPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

The gene encoding a CRPI can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified DNA encoding a CRPI of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies that specifically recognize a CRPI. A radiolabelled cDNA encoding a CRPI can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the DNA fragments encoding a CRPI from among other genomic DNA fragments.

Alternatives to isolating genomic DNA encoding a CRPI include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the CRPI. For example, RNA for

cDNA cloning of the gene encoding a CRPI can be isolated from cells which express the CRPI. Those skilled in the art will understand from the present description that other methods may be used and are within the scope of the invention.

Any suitable eukaryotic cell can serve as the nucleic acid source for the molecular cloning of the gene encoding a CRPI. The nucleic acid sequences encoding the CRPI can be isolated from vertebrate, mammalian, primate, human, porcine, bovine, feline, avian, equine, canine or murine sources. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (*See, e.g.,* Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences.

The identified and isolated gene or cDNA can then be inserted into any suitable cloning vector. A large number of vector-host systems known in the art may be used. As those skilled in the art will appreciate, the only limitation is that the vector system chosen be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene) or modified viruses such as adenoviruses, adeno-associated viruses or retroviruses. The insertion into a cloning vector can be accomplished, for example, by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and the gene encoding a CRPI may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated gene encoding the CRPI, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The nucleotide sequences of the present invention include nucleotide sequences encoding amino acid sequences with substantially the same amino acid sequences as native CRPIs, nucleotide sequences encoding amino acid sequences with functionally equivalent amino acids, nucleotide sequences encoding CRPIs, a fragments of CRPIs, CRPI-related polypeptides, or fragments of CRPI-related polypeptides.

In a specific embodiment, an isolated nucleic acid molecule encoding a CRPI-related polypeptide can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of a CRPI such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Standard techniques known to those of skill in the art can be used to introduce mutations, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed and the activity of the protein can be determined.

5.7 Expression of DNA Encoding CRPIs

The nucleotide sequence coding for a CRPI, a CRPI analog, a CRPI-related peptide, or a fragment or other derivative of any of the foregoing, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native gene encoding the CRPI or its flanking regions, or the native gene encoding the CRPI-related polypeptide or its flanking regions. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, a nucleotide sequence encoding a human gene (or a nucleotide sequence encoding a functionally active portion of a huma CRPI) is expressed. In yet another embodiment, a fragment of a CRPI comprising a domain of the CRPI is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a CRPI or fragment thereof may be regulated by a second nucleic acid sequence so that the CRPI or fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a CRPI may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding a CRPI or a CRPI-related polypeptide include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter

(Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, Proc. Nat. Acad. Sci. USA 89:5547-5551); prokaryotic expression vectors such as the _-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al., 1999, Gen. Virol. 80:571-83); brain-derived neurotrophic factor

(BDNF) gene control region which is active in neuronal cells (Tabuchi et al., 1998, Biochem. Biophysic. Res. Com. 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al., 1999, Braz J Med Biol Res 32(5):619-631; Morelli et al., 1999, Gen. Virol. 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a CRPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a CRPI or a CRPI-related polypeptide coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the CRPI product or CRPI-related polypeptide from the subclone in the correct reading frame.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the CRPI coding sequence or CRPI-related polypeptide coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (*e.g.*, see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, *Methods in Enzymol.* 153:51-544).

Expression vectors containing inserts of a gene encoding a CRPI or a CRPI-related polypeptide can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c)

expression of inserted sequences. In the first approach, the presence of a gene encoding a CRPI inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding a CRPI. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a gene encoding a CRPI in the vector. For example, if the gene encoding the CRPI is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the CRPI insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (*i.e.*, CRPI) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the CRPI in *in vitro* assay systems, *e.g.*, binding with anti-CRPI antibody.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered CRPI or CRPI-related polypeptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, HEK293, 3T3, WI38, and in particular, cardiac muscle cell lines, and normal human cell lines such as. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than

using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

In other specific embodiments, the CRPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence). For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system. An increase in the half-life *in vivo* and facilitated purification has been shown for chimeric proteins consisting of the first two domains of the

human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. See, *e.g.*, EP 394,827; Traunecker *et al.*, *Nature*, 331:84-86 (1988). Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (*e.g.*, insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, *e.g.*, PCT publications WO 96/22024 and WO 99/04813).

Nucleic acids encoding a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide can fused to an epitope tag (*e.g.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht *et al.*, 1991, *Proc. Natl. Acad. Sci.* USA 88:8972-897).

Fusion proteins can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, a fusion protein may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.8 Domain Structure of CRPIs

Domains of some CRPIs are known in the art and have been described in the scientific literature. Moreover, domains of a CRPI can be identified using techniques known to those of skill in the art. For example, one or more domains of a CRPI can be identified by using one or more of the following programs: ProDom, TMpred, and SAPS. ProDom compares the amino acid sequence of a polypeptide to a database of compiled domains (*see, e.g.*, http://www.toulouse.inra.fr/prodom.html; Corpet F., Gouzy J. & Kahn D., 1999, Nucleic Acids Res., 27:263-267). TMpred predicts membrane-spanning regions of a polypeptide and their orientation. This program uses an algorithm that is based on the statistical analysis of TMbase, a database of naturally occuring transmembrane proteins (*see, e.g.*, http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann & Stoffel. (1993) "TMbase - A database of membrane spanning proteins segments." Biol. Chem. Hoppe-Seyler 347,166). The SAPS program analyzes polypeptides for statistically significant features like charge-clusters, repeats, hydrophobic regions, compositional domains (see, *e.g.*, Brendel et al., 1992, Proc. Natl. Acad. Sci. USA

89: 2002-2006). Thus, based on the present description, the skilled artisan can identify domains of a CRPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of a CRPI fragment that retains the enzymatic or binding activity of the CRPI.

Based on the present description, the skilled artisan can identify domains of a CRPI having enzymatic or binding activity, and further can identify nucleotide sequences encoding such domains. These nucleotide sequences can then be used for recombinant expression of CRPI fragments that retain the enzymatic or binding activity of the CRPI.

In one embodiment, a CRPI has an amino acid sequence sufficiently similar to an identified domain of a known polypeptide. As used herein, the term "sufficiently similar" refers to a first amino acid or nucleotide sequence which contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have or encode a common structural domain or common functional activity or both.

A CRPI domain can be assessed for its function using techniques well known to those of skill in the art. For example, a domain can be assessed for its kinase activity or for its ability to bind to DNA using techniques known to the skilled artisan. Kinase activity can be assessed, for example, by measuring the ability of a polypeptide to phosphorylate a substrate. DNA binding activity can be assessed, for example, by measuring the ability of a polypeptide to bind to a DNA binding element in a electromobility shift assay. In a preferred embodiment, the function of a domain of a CRPI is determined using an assay described in one or more of the references identified in Table X, *infra*.

5.9 Production of Antibodies to CRPIs

According to the invention a CRPI, CRPI analog, CRPI-related protein or a fragment or derivative of any of the foregoing may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such immunogens can be isolated by any convenient means, including the methods described above. Antibodies of the invention include, but are not limited to polyclonal, monoclonal, bispecific, humanized or chimeric antibodies, single chain antibodies, Fab fragments and F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds an antigen. The immunoglobulin molecules of the invention can be of any class (*e.g.*, IgG, IgE, IgM, IgD and IgA) or subclass of immunoglobulin molecule.

In one embodiment, antibodies that recognize gene products of genes encoding CRPIs are publicly available. For example, antibodies that recognize these CRPIs and/or their isoforms include antibodies which can be purchased from commercial sources as described above. In another embodiment, methods known to those skilled in the art are used to produce antibodies that recognize a CRPI, a CRPI analog, a CRPI-related polypeptide, or a derivative or fragment of any of the foregoing.

In one embodiment of the invention, antibodies to a specific domain of a CRPI are produced. In a specific embodiment, hydrophilic fragments of a CRPI are used as immunogens for antibody production.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a CRPI, one may assay generated hybridomas for a product which binds to a CRPI fragment containing such domain. For selection of an antibody that specifically binds a first CRPI homolog but which does not specifically bind to (or binds less avidly to) a second CRPI homolog, one can select on the basis of positive binding to the first CRPI homolog and a lack of binding to (or reduced binding to) the second CRPI homolog. Similarly, for selection of an antibody that specifically binds a CRPI but which does not specifically bind to (or binds less avidly to) a different isoform of the same protein (such as a different glycoform having the same core peptide as the CRPI), one can select on the basis of positive binding to the

CRPI and a lack of binding to (or reduced binding to) the different isoform (*e.g.*, a different glycoform). Thus, the present invention provides an antibody (preferably a monoclonal antibody) that binds with greater affinity (preferably at least 2-fold, more preferably at least 5-fold still more preferably at least 10-fold greater affinity) to a CRPI than to a different isoform or isoforms (*e.g.*, glycoforms) of the CRPI.

Polyclonal antibodies which may be used in the methods of the invention are heterogeneous populations of antibody molecules derived from the sera of immunized animals. Unfractionated immune serum can also be used. Various procedures known in the art may be used for the production of polyclonal antibodies to a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide. In a particular embodiment, rabbit polyclonal antibodies to an epitope of a CRPI or a CRPI-related polypeptide can be obtained. For example, for the production of polyclonal or monoclonal antibodies, various host animals can be immunized by injection with the native or a synthetic (*e.g.*, recombinant) version of a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide, including but not limited to rabbits, mice, rats, etc. The Preferred Technology described herein provides isolated CRPIs suitable for such immunization. If the CRPI is purified by gel electrophoresis, the CRPI can be used for immunization with or without prior extraction from the polyacrylamide gel. Various adjuvants may be used to enhance the immunological response, depending on the host species, including, but not limited to, complete or incomplete Freund's adjuvant, a mineral gel such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyol, a polyanion, a peptide, an oil emulsion, keyhole limpet hemocyanin, dinitrophenol, and an adjuvant such as BCG (bacille Calmette-Guerin) or corynebacterium parvum. Additional adjuvants are also well known in the art.

For preparation of monoclonal antibodies (mAbs) directed toward a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, or a fragment of a CRPI-related polypeptide, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any

immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAbs of the invention may be cultivated *in vitro* or *in vivo*. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing known technology (PCT/US90/02545, incorporated herein by reference).

The monoclonal antibodies include but are not limited to human monoclonal antibodies and chimeric monoclonal antibodies (*e.g.*, human-mouse chimeras). A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, *e.g.*, Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.)

Chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al., 1988, Science 240:1041-1043; Liu et al., 1987, Proc. Natl. Acad. Sci. USA 84:3439-3443; Liu et al., 1987, J. Immunol. 139:3521-3526; Sun et al., 1987, Proc. Natl. Acad. Sci. USA 84:214-218; Nishimura et al., 1987, Canc. Res. 47:999-1005; Wood et al., 1985, Nature 314:446-449; and Shaw et al., 1988, J. Natl. Cancer Inst. 80:1553-1559; Morrison, 1985, Science 229:1202-1207; Oi et al., 1986, Bio/Techniques 4:214; U.S. Patent 5,225,539; Jones et al., 1986, Nature 321:552-525; Verhoeyan et al. (1988) Science 239:1534; and Beidler et al., 1988, J. Immunol. 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Such antibodies can be produced using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a CRPI of the invention. Monoclonal antibodies directed against

the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, Int. Rev. Immunol. 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al. (1994) Bio/technology 12:899-903).

The antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labelled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., J. Immunol. Methods 182:41-50 (1995); Ames et al., J. Immunol. Methods 184:177-186 (1995); Kettleborough et al., Eur. J. Immunol. 24:952-958 (1994); Persic et al., Gene 187 9-18 (1997); Burton et al., Advances in Immunology 57:191-280 (1994); PCT Application No. PCT/GB91/01134; PCT Publications WO 90/02809; WO 91/10737;

WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al., Methods in Enzymology* 203:46-88 (1991); Shu *et al., PNAS* 90:7995-7999 (1993); and Skerra *et al., Science* 240:1038-1040 (1988).

The invention further provides for the use of bispecific antibodies, which can be made by methods known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., 1983, Nature 305:537-539). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., 1991, EMBO J. 10:3655-3659 .

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least

one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published March 3,1994. For further details for generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology,1986, 121:210.

The invention provides functionally active fragments, derivatives or analogs of the anti-CRPI immunoglobulin molecules. Functionally active means that the fragment, derivative or analog is able to elicit anti-anti-idiotype antibodies (*i.e.*, tertiary antibodies) that recognize the same antigen that is recognized by the antibody from which the fragment, derivative or analog is derived. Specifically, in a preferred embodiment the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art.

The present invention provides antibody fragments such as, but not limited to, $F(ab')_2$ fragments and Fab fragments. Antibody fragments which recognize specific epitopes may be generated by known techniques. $F(ab')_2$ fragments consist of the variable region, the light chain constant region and the CH1 domain of the heavy chain and are generated by pepsin digestion of the antibody molecule. Fab

fragments are generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. The invention also provides heavy chain and light chain dimers of the antibodies of the invention, or any minimal fragment thereof such as Fvs or single chain antibodies (SCAs) (*e.g.*, as described in U.S. Patent 4,946,778; Bird, 1988, Science 242:423-42; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-54), or any other molecule with the same specificity as the antibody of the invention. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may be used (Skerra et al., 1988, Science 242:1038-1041).

In other embodiments, the invention provides fusion proteins of the immunoglobulins of the invention (or functionally active fragments thereof), for example in which the immunoglobulin is fused via a covalent bond (*e.g.*, a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably at least 10, 20 or 50 amino acid portion of the protein) that is not the immunoglobulin. Preferably the immunoglobulin, or fragment thereof, is covalently linked to the other protein at the N-terminus of the constant domain. As stated above, such fusion proteins may facilitate purification, increase half-life *in vivo*, and enhance the delivery of an antigen across an epithelial barrier to the immune system.

The immunoglobulins of the invention include analogs and derivatives that are either modified, *i.e*, by the covalent attachment of any type of molecule as long as such covalent attachment that does not impair immunospecific binding. For example, but not by way of limitation, the derivatives and analogs of the immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the CRPIs of the invention, *e.g.*, for imaging these

proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

5.10 Expression Of Antibodies

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression techniques.

Recombinant expression of antibodies, or fragments, derivatives or analogs thereof, requires construction of a nucleic acid that encodes the antibody. If the nucleotide sequence of the antibody is known, a nucleic acid encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al., 1994, BioTechniques 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, the nucleic acid encoding the antibody may be obtained by cloning the antibody. If a clone containing the nucleic acid encoding the particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the antibody may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the antibody) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence.

If an antibody molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an antibody), antibodies specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies. Alternatively, a clone encoding at least the Fab portion of the antibody may be obtained by screening Fab expression libraries (*e.g.*, as described in Huse et al., 1989, *Science* 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, *e.g.*, Clackson et al., 1991, Nature 352:624; Hane et al., 1997 Proc. Natl. Acad. Sci. USA 94:4937).

Once a nucleic acid encoding at least the variable domain of the antibody molecule is obtained, it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the antibody molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available. Then, the nucleic acid encoding the antibody can be used to introduce the nucleotide substitution(s) or deletion(s) necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydyl group. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a nucleotide sequence, for example, but not limited to, chemical mutagenesis, in vitro site directed mutagenesis (Hutchinson et al., 1978, J. Biol. Chem. 253:6551), PCT based methods, etc.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:851-855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human antibody constant region, *e.g.*, humanized antibodies.

Once a nucleic acid encoding an antibody molecule of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protein of the invention by expressing nucleic acid containing the antibody molecule sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing an antibody molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al. (1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory,

Cold Spring Harbor, NY) and Ausubel et al. (eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention.

The host cells used to express a recombinant antibody of the invention may be either bacterial cells such as *Escherichia coli,* or, preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 198, Gene 45:101; Cockett et al., 1990, Bio/Technology 8:2).

A variety of host-expression vector systems may be utilized to express an antibody molecule of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibody molecule of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, HEK293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions comprising an antibody molecule,

vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). In mammalian host cells, a number of viral-based expression systems (*e.g.*, an adenovirus expression system) may be utilized.

As discussed above, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein.

For long-term, high-yield production of recombinant antibodies, stable expression is preferred. For example, cells lines that stably express an antibody of interest can be produced by transfecting the cells with an expression vector comprising the nucleotide sequence of the antibody and the nucleotide sequence of a selectable (*e.g.*, neomycin or hygromycin), and selecting for expression of the selectable marker. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

The expression levels of the antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based

on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., 1983, Mol. Cell. Biol. 3:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, Nature 322:52; Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once the antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an antibody molecule, for example, by chromatography (*e.g.*, ion exchange chromatography, affinity chromatography such as with protein A or specific antigen, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni2+ nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

5.11 Conjugated Antibodies

In a preferred embodiment, anti-CRPI antibodies or fragments thereof are conjugated to a diagnostic or therapeutic moiety. The antibodies can be used for diagnosis or to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include ^{125}I, ^{131}I, ^{111}In and ^{99}Tc.

An anti-CRPI antibodies or fragments thereof can be conjugated to a therapeutic agent or drug moiety to modify a given biological response. The therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, -interferon, -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.*, angiostatin or endostatin; or, a biological response modifier such as a lymphokine, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-blood), granulocyte colony stimulating factor (G-blood), nerve growth factor (NGF) or other growth factor.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For

Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

An antibody with or without a therapeutic moiety conjugated to it can be used as a therapeutic that is administered alone or in combination with cytotoxic factor(s) and/or cytokine(s).

5.12 Diagnosis of Cardiac Response

In accordance with the present invention, test samples of blood, cerebrospinal fluid, serum, plasma or urine obtained from a subject suspected of having or known to have cardiac response can be used for diagnosis or monitoring.

In one embodiment, a decreased abundance of one or more CRFs or CRPIs (or any combination of them) in a test sample relative to a control sample (from a subject or subjects free from cardiac response) or a previously determined reference range indicates the presence of cardiac response; CRFs and CRPIs suitable for this purpose are identified in Tables I, III(b), V and VII, respectively, as described in detail above.

In another embodiment of the invention, an increased abundance of one or more CRFs or CRPIs (or any combination of them) in a test sample compared to a control sample or a previously determined reference range indicates the presence of cardiac response; CRFs and CRPIs suitable for this purpose are identified in Tables II, III(a), and VI, respectively, as described in detail above.

In another embodiment, the relative abundance of one or more CRFs or CRPIs (or any combination of them) in a test sample compared to a control sample or a previously determined reference range indicates a subtype of cardiac response (*e.g.*, familial or sporadic cardiac response). In yet another embodiment, the relative abundance of one or more CRFs or CRPIs (or any combination of them) in a test

sample relative to a control sample or a previously determined reference range indicates the degree or severity of cardiac response. In any of the aforesaid methods, detection of one or more CRPIs described herein may optionally be combined with detection of one or more additional biomarkers for cardiac response including, but not limited to apolipoprotein A1 (Apo E). Any suitable method in the art can be employed to measure the level of CRFs and CRPIs, including but not limited to the Preferred Technology described herein, kinase assays, immunoassays to detect and/or visualize the CRPI (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.). In cases where a CRPI has a known function, an assay for that function may be used to measure CRPI expression. In a further embodiment, a decreased abundance of mRNA including one or more CRPIs identified in Tables V and VII (or any combination of them) in a test sample relative to a control sample or a previously determined reference range indicates the presence of cardiac response. In yet a further embodiment, an increased abundance of mRNA encoding one or more CRPIs identified in Table VI (or any combination of them) in a test sample relative to a control sample or previously determined reference range indicates the presence of cardiac response. Any suitable hybridization assay can be used to detect CRPI expression by detecting and/or visualizing mRNA encoding the CRPI (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

In another embodiment of the invention, labeled antibodies, derivatives and analogs thereof, which specifically bind to a CRPI can be used for diagnostic purposes to detect, diagnose, or monitor cardiac response. Preferably, cardiac response is detected in an animal, more preferably in a mammal and most preferably in a human.

5.13 Screening Assays

The invention provides methods for identifying agents (*e.g.*, drug candidates or test compounds) or environmental factors that bind to a CRPI or have a stimulatory or inhibitory effect on the expression or activity of a CRPI. The invention also provides methods of identifying agents, candidate compounds, test compounds or environmental factors that bind to a CRPI-related polypeptide or a CRPI fusion protein or have a stimulatory or inhibitory effect on the expression or activity of a CRPI-related polypeptide or a CRPI fusion protein. In addition, the invention provides methods for identifying agents or environmental factors that do

not to a CRPI or do not have a stimulatory or inhibitory effect on the expression or activity of a CRPI. The invention also provides methods of identifying agents, candidate compounds, test compounds or environmental factors that do not bind to a CRPI-related polypeptide or a CRPI fusion protein or do not have a stimulatory or inhibitory effect on the expression or activity of a CRPI-related polypeptide or a CRPI fusion protein. The following examples of embodiments within the scope of the present invention are expressed as screening for the presence of binding of a CRPI, or the presence of an effect on the expression or activity of a CRPI, however, it will be clear to one of skill in the art that the examples are readily modified so as to be applicable to screening for the absence of such binding or effect.

Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules and other drugs. Agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, Anticancer Drug Des. 12:145; U.S. Patent No. 5,738,996; and U.S. Patent No.5,807,683, each of which is incorporated herein in its entirety by reference).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al., 1993, Proc. Natl. Acad. Sci. USA 90:6909; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al., 1994, J. Med. Chem. 37:2678; Cho et al., 1993, Science 261:1303; Carrell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al., 1994, J. Med. Chem. 37:1233, each of which is incorporated herein in its entirety by reference.

Libraries of compounds may be presented, *e.g.*, presented in solution (*e.g.*, Houghten, 1992, Bio/Techniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al., 1992, Proc. Natl. Acad. Sci. USA 89:1865-1869) or phage (Scott and Smith, 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla et

al., 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; and Felici, 1991, J. Mol. Biol. 222:301-310), each of which is incorporated herein in its entirety by reference.

In one embodiment, agents that interact with (*i.e.*, bind to) a CRPI, a CRPI fragment (e.g. a functionally active fragment), a CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein are identified in a cell-based assay system. In accordance with this embodiment, cells expressing a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein are contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the CRPI is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. The cell, for example, can be of prokaryotic origin (*e.g.*, *E. coli*) or eukaryotic origin (*e.g.*, yeast or mammalian). Further, the cells can express the CRPI, fragment of the CRPI, CRPI-related polypeptide, a fragment of the CRPI-related polypeptide, or a CRPI fusion protein endogenously or be genetically engineered to express the CRPI, fragment of the CRPI, CRPI-related polypeptide, a fragment of the CRPI-related polypeptide, or a CRPI fusion protein. In certain instances, the CRPI, fragment of the CRPI, CRPI-related polypeptide, a fragment of the CRPI-related polypeptide, or a CRPI fusion protein or the candidate compound is labeled, for example with a radioactive label (such as ^{32}P, ^{35}S or ^{125}I) or a fluorescent label (such as fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde or fluorescamine) to enable detection of an interaction between a CRPI and a candidate compound. The ability of the candidate compound to interact directly or indirectly with a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein can be determined by methods known to those of skill in the art. For example, the interaction between a candidate compound and a CRPI, a fragment of a CRPI, a CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein can be determined by flow cytometry, a scintillation assay, immunoprecipitation or western blot analysis.

In another embodiment, agents that interact with (*i.e.*, bind to) a CRPI, a CRPI fragment (*e.g.*, a functionally active fragment) a CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein are identified in a cell-free assay system. In accordance with this embodiment, a native or recombinant CRPI or fragment thereof, or a native or recombinant CRPI-related polypeptide or

fragment thereof, or a CRPI-fusion protein or fragment thereof, is contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the CRPI or CRPI-related polypeptide, or CRPI fusion protein is determined. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. Preferably, the CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI-fusion protein is first immobilized, by, for example, contacting the CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein with an immobilized antibody which specifically recognizes and binds it, or by contacting a purified preparation of the CRPI, CRPI fragment, CRPI-related polypeptide, fragment of a CRPI-related polypeptide, or a CRPI fusion protein with a surface designed to bind proteins. The CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein may be partially or completely purified (*e.g.*, partially or completely free of other polypeptides) or part of a cell lysate. Further, the CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide may be a fusion protein comprising the CRPI or a biologically active portion thereof, or CRPI-related polypeptide and a domain such as glutathionine-S-transferase. Alternatively, the CRPI, CRPI fragment, CRPI-related polypeptide, fragment of a CRPI-related polypeptide or CRPI fusion protein can be biotinylated using techniques well known to those of skill in the art (*e.g.*, biotinylation kit, Pierce Chemicals; Rockford, IL). The ability of the candidate compound to interact with a CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein can be can be determined by methods known to those of skill in the art.

In another embodiment, a cell-based assay system is used to identify agents that bind to or modulate the activity of a protein, such as an enzyme, or a biologically active portion thereof, which is responsible for the production or degradation of a CRPI or is responsible for the post- translational modification of a CRPI. In a primary screen, a plurality (*e.g.*, a library) of compounds (*e.g.,* drug candidates) are contacted with cells that naturally or recombinantly express: (i) a CRPI, an isoform of a CRPI, a CRPI homolog a CRPI-related polypeptide, a CRPI fusion protein, or a biologically active fragment of any of the foregoing; and (ii) a protein that is responsible for processing of the CRPI, CRPI isoform, CRPI homolog, CRPI-related polypeptide, CRPI fusion protein, or fragment in order to

identify compounds that modulate the production, degradation, or post-translational modification of the CRPI, CRPI isoform, CRPI homolog, CRPI-related polypeptide, CRPI fusion protein or fragment. If desired, compounds identified in the primary screen can then be assayed in a secondary screen against cells naturally or recombinantly expressing the specific CRPI of interest. The ability of the candidate compound to modulate the production, degradation or post-translational modification of a CRPI, isoform, homolog, CRPI-related polypeptide, or CRPI fusion protein can be determined by methods known to those of skill in the art, including without limitation, flow cytometry, a scintillation assay, immunoprecipitation and western blot analysis.

In another embodiment, agents that competitively interact with (*i.e.*, bind to) a CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein are identified in a competitive binding assay. In accordance with this embodiment, cells expressing a CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein are contacted with a candidate compound and a compound known to interact with the CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide or a CRPI fusion protein; the ability of the candidate compound to competitively interact with the CRPI, CRPI fragment, CRPI-related polypeptide, fragment of a CRPI-related polypeptide, or a CRPI fusion protein is then determined. Alternatively, agents that competitively interact with (*i.e.*, bind to) a CRPI, CRPI fragment, CRPI-related polypeptide or fragment of a CRPI-related polypeptide are identified in a cell-free assay system by contacting a CRPI, CRPI fragment, CRPI-related polypeptide, fragment of a CRPI-related polypeptide, or a CRPI fusion protein with a candidate compound and a compound known to interact with the CRPI, CRPI-related polypeptide or CRPI fusion protein. As stated above, the ability of the candidate compound to interact with a CRPI, CRPI fragment, CRPI-related polypeptide, a fragment of a CRPI-related polypeptide, or a CRPI fusion protein can be determined by methods known to those of skill in the art. These assays, whether cell-based or cell-free, can be used to screen a plurality (*e.g.*, a library) of candidate compounds.

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression of a CRPI, or a CRPI-related polypeptide are identified by contacting cells (*e.g.*, cells of prokaryotic origin or eukaryotic origin) expressing the CRPI, or CRPI-related polypeptide with a candidate compound or a control

compound (*e.g.*, phosphate buffered saline (PBS)) and determining the expression of the CRPI, CRPI-related polypeptide, or CRPI fusion protein, mRNA encoding the CRPI, or mRNA encoding the CRPI-related polypeptide. The level of expression of a selected CRPI, CRPI-related polypeptide, mRNA encoding the CRPI, or mRNA encoding the CRPI-related polypeptide in the presence of the candidate compound is compared to the level of expression of the CRPI, CRPI-related polypeptide, mRNA encoding the CRPI, or mRNA encoding the CRPI-related polypeptide in the absence of the candidate compound (*e.g.*, in the presence of a control compound). The candidate compound can then be identified as a modulator of the expression of the CRPI, or a CRPI-related polypeptide based on this comparison. For example, when expression of the CRPI or mRNA is significantly greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of expression of the CRPI or mRNA. Alternatively, when expression of the CRPI or mRNA is significantly less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of the expression of the CRPI or mRNA. The level of expression of a CRPI or the mRNA that encodes it can be determined by methods known to those of skill in the art. For example, mRNA expression can be assessed by Northern blot analysis or RT-PCR, and protein levels can be assessed by western blot analysis.

In another embodiment, agents that modulate the activity of a CRPI, or a CRPI-related polypeptide are identified by contacting a preparation containing the CRPI or CRPI-related polypeptide, or cells (*e.g.*, prokaryotic or eukaryotic cells) expressing the CRPI or CRPI-related polypeptide with a test compound or a control compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the CRPI or CRPI-related polypeptide. The activity of a CRPI or a CRPI-related polypeptide can be assessed by detecting induction of a cellular signal transduction pathway of the CRPI or CRPI-related polypeptide (*e.g.* , intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the target on a suitable substrate, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a CRPI or a CRPI-related polypeptide and is operably linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, cellular differentiation, or cell proliferation. Based on the present description, techniques known to those of skill in the art can be used for measuring these activities (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference). The

candidate compound can then be identified as a modulator of the activity of a CRPI or CRPI-related polypeptide by comparing the effects of the candidate compound to the control compound. Suitable control compounds include phosphate buffered saline (PBS) and normal saline (NS).

In another embodiment, agents that modulate (*i.e.*, upregulate or downregulate) the expression, activity or both the expression and activity of a CRPI or CRPI-related polypeptide are identified in an animal model. Examples of suitable animals include, but are not limited to, mice, rats, rabbits, monkeys, guinea pigs, dogs and cats. Preferably, the animal used represent a model of cardiac response. In accordance with this embodiment, the test compound or a control compound is administered (*e.g.*, orally, rectally or parenterally such as intraperitoneally or intravenously) to a suitable animal and the effect on the expression, activity or both expression and activity of the CRPI or CRPI-related polypeptide is determined. Changes in the expression of a CRPI or CRPI-related polypeptide can be assessed by the methods outlined above.

In yet another embodiment, a CRPI or CRPI-related polypeptide is used as a "bait protein" in a two-hybrid assay or three hybrid assay to identify other proteins that bind to or interact with a CRPI or CRPI-related polypeptide (*see, e.g.*, U.S. Patent No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Bio/Techniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and PCT Publication No. WO 94/10300). As those skilled in the art will appreciate, such binding proteins are also likely to be involved in the propagation of signals by the CRPIs of the invention as, for example, upstream or downstream elements of a signaling pathway involving the CRPIs of the invention.

Table X enumerates scientific publications describing suitable assays for detecting or quantifying enzymatic or binding activity of a CRPI, a CRPI analog, a CRPI-related polypeptide, or a fragment of any of the foregoing. Each such reference is hereby incorporated in its entirety. In a preferred embodiment, as assay referenced in Table X is used in the screens and assays described herein, for example to screen for or identify a compound that modulates the activity of (or that modulates both the expression and activity of) a CRPI, CRPI analog, or CRPI-related polypeptide, a fragment of any of the foregoing.

Table X. Scientific publications

Table X CRF	CRPI	Assay Reference: Scientific Publication
CRF-1, CRF-2, CRF-14	CRPI-1, CRF-2, CRPI-14	"Rational design of potent human transthyretin amyloid disease inhibitors." Nat Struct Biol. (2000) Apr;7(4):312-21. "Structure-Based Design of N-Phenyl Phenoxazine Transthyretin Amyloid Fibril Inhibitors" J. Am. Chem. Soc.(2000) 122, 2178-2192,

More particularly, in one aspect, the invention provides methods for the identification of agents which will not have an effect on the expression or activity of a CRPI, CRPI-related polypeptide or CRPI fusion protein, and as such will not induce a cardiac response. When such agents are drug candidiates they can be propgressed into development with a greater level of confidence that they will not produce unwanted cardiac responses when administered clinically.

This aspect of the invention allows for toxicity screening to be carried out at a much earlier stage. In particular, it can show whether agent will or will not induce cardiac response. In relation to the screening of agents for their potential to induce an unwanted cardiac response, the term "agent" is used herein to describe a wide variety of physical, chemical or biological factors. For example, physical agents include, without limitation, the diet of a subject, a change in temperature or humidity, exposure to ultraviolet radiation and the like. Biological and chemical agents include exogenous factors such as pharmaceutical compounds (including candidate compounds and test compounds), toxic compounds, proteins, peptides, chemical compositions, natural pathogens, such as microbial agents including bacteria, viruses and lower eukaryotic cells such as fungi, yeast and simple multicellular organisms, as well as endogenous factors which occur naturally in the body, including, without limitation, hormones, enzymes, receptors, ligands and the like, which may or may not be recombinant.

This invention further provides novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

5.14 Therapeutic Uses of CRPIs

The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound. Such compounds include but are not limited to: CRPIs, CRPI analogs, CRPI-related polypeptides and derivatives (including fragments) thereof; antibodies to the foregoing; nucleic acids encoding CRPIs, CRPI analogs, CRPI-related polypeptides and fragments thereof; antisense nucleic acids to a gene encoding a CRPI or CRPI-related polypeptide; and modulator (*e.g.*, agonists and antagonists) of a gene encoding a CRPI or CRPI-related polypeptide. An important feature of the present invention is the identification of genes encoding CRPIs involved in cardiac response. Cardiac response can be treated (e.g. to ameliorate symptoms or to retard onset or progression) or prevented by administration of a therapeutic compound that promotes function or expression of one or more CRPIs that are decreased in the blood of cardiac response subjects having cardiac response, or by administration of a therapeutic compound that reduces function or expression of one or more CRPIs that are increased in the blood of subjects having cardiac response.

In one embodiment, one or more antibodies each specifically binding to a CRPI are administered alone or in combination with one or more additional therapeutic compounds or treatments. Examples of such therapeutic compounds or treatments include, but are not limited to, interferon, cholestyrene, limivudine.

Preferably, a biological product such as an antibody is allogeneic to the subject to which it is administered. In a preferred embodiment, a human CRPI or a human CRPI-related polypeptide, a nucleotide sequence encoding a human CRPI or a human CRPI-related polypeptide, or an antibody to a human CRPI or a human CRPI-related polypeptide, is administered to a human subject for therapy (e.g. to ameliorate symptoms or to retard onset or progression) or prophylaxis.

5.14.1 Treatment And Prevention Of Cardiac response

Cardiac response is treated or prevented by administration to a subject suspected of having or known to have cardiac response or to be at risk of developing cardiac response of a compound that modulates (*i.e.*, increases or decreases) the level or activity (*i.e.*, function) of one or more CRPIs -- or the level of one or more CRFs -- that are differentially present in the blood of subjects having cardiac response compared with blood of subjects free from cardiac response. In one embodiment, cardiac response is treated or prevented by administering to a subject

suspected of having or known to have cardiac response or to be at risk of developing cardiac response a compound that upregulates (*i.e.*, increases) the level or activity (*i.e.*, function) of one or more CRPIs -- or the level of one or more CRFs -- that are decreased in the blood of subjects having cardiac response. In another embodiment, a compound is administered that upregulates the level or activity (*i.e.*, function) of one or more CRPIs -- or the level of one or more CRFs -- that are increased in the blood of subjects having cardiac response. Examples of such a compound include but are not limited to: CRPIs, CRPI fragments and CRPI-related polypeptides; nucleic acids encoding a CRPI, a CRPI fragment and a CRPI-related polypeptide (*e.g.*, for use in gene therapy); and, for those CRPIs or CRPI-related polypeptides with enzymatic activity, compounds or molecules known to modulate that enzymatic activity. Other compounds that can be used, *e.g.*, CRPI agonists, can be identified using *in vitro* assays.

Cardiac response is also treated or prevented by administration to a subject suspected of having or known to have cardiac response or to be at risk of developing cardiac response of a compound that downregulates the level or activity of one or more CRPIs -- or the level of one or more CRFs -- that are increased in the blood of subjects having cardiac response. In another embodiment, a compound is administered that downregulates the level or activity of one or more CRPIs -- or the level of one or more CRFs -- that are decreased in the blood of subjects having cardiac response. Examples of such a compound include, but are not limited to, CRPI antisense oligonucleotides, ribozymes, antibodies directed against CRPIs, and compounds that inhibit the enzymatic activity of a CRPI. Other useful compounds *e.g.*, CRPI antagonists and small molecule CRPI antagonists, can be identified using *in vitro* assays.

In a preferred embodiment, therapy or prophylaxis is tailored to the needs of an individual subject. Thus, in specific embodiments, compounds that promote the level or function of one or more CRPIs, or the level of one or more CRFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have cardiac response, in whom the levels or functions of said one or more CRPIs, or levels of said one or more CRFs, are absent or are decreased relative to a control or normal reference range. In further embodiments, compounds that promote the level or function of one or more CRPIs, or the level of one or more CRFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have cardiac response in whom the levels or functions of said one or more

CRPIs, or levels of said one or more CRFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function of one or more CRPIs, or the level of one or more CRFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have cardiac response in whom the levels or functions of said one or more CRPIs, or levels of said one or more CRFs, are increased relative to a control or to a reference range. In further embodiments, compounds that decrease the level or function of one or more CRPIs, or the level of one or more CRFs, are therapeutically or prophylactically administered to a subject suspected of having or known to have cardiac response in whom the levels or functions of said one or more CRPIs, or levels of said one or more CRFs, are decreased relative to a control or to a reference range. The change in CRPI function or level, or CRF level, due to the administration of such compounds can be readily detected, *e.g.*, by obtaining a sample (*e.g.*, a sample of blood, or urine or a tissue sample such as a biopsy tissue) and assaying *in vitro* the levels of said CRFs or the levels or activities of said CRPIs, or the levels of mRNAs encoding said CRPIs. or any combination of the foregoing. Such assays can be performed before and after the administration of the compound as described herein.

The compounds of the invention include but are not limited to any compound, *e.g.*, a small organic molecule, protein, peptide, antibody, nucleic acid, etc. that restores the cardiac response CRPI or CRF profile towards normal.

5.14.2 Gene Therapy

In a specific embodiment, nucleic acids comprising a sequence encoding a CRPI, a CRPI fragment, CRPI-related polypeptide or fragment of a CRPI-related polypeptide, are administered to promote CRPI function by way of gene therapy. Gene therapy refers to administration to a subject of an expressed or expressible nucleic acid. In this embodiment, the nucleic acid produces its encoded polypeptide that mediates a therapeutic effect by promoting CRPI function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem.

62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the compound comprises a nucleic acid encoding a CRPI or fragment or chimeric protein thereof, said nucleic acid being part of an expression vector that expresses a CRPI or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the CRPI coding region, said promoter being inducible or constitutive (and, optionally, tissue-specific). In another particular embodiment, a nucleic acid molecule is used in which the CRPI coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the CRPI nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a subject may be direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid-carrying vector; this approach is known as *in vivo* gene therapy. Alternatively, delivery of the nucleic acid into the subject may be indirect, in which case cells are first transformed with the nucleic acid *in vitro* and then transplanted into the subject; this approach is known as *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286); by direct injection of naked DNA; by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); by coating with lipids, cell-surface receptors or transfecting agents; by encapsulation in liposomes, microparticles or microcapsules; by administering it in linkage to a peptide which is known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic acid-ligand complex can be formed in

which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector that contains a nucleic acid encoding a CRPI is used. For example, a retroviral vector can be used (see Miller et al., 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid encoding the CRPI to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a subject. More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene

therapy can be found in Rosenfeld et al., 1991, Science 252:431-434; Rosenfeld et al., 1992, Cell 68:143-155; Mastrangeli et al., 1993, J. Clin. Invest. 91:225-234; PCT Publication WO94/12649; and Wang, et al., 1995, Gene Therapy 2:775-783.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a subject by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the subject. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, the condition of the subject, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to

neuronal cells, glial cells (*e.g.*, oligodendrocytes or astrocytes), epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood or fetal liver.

In a preferred embodiment, the cell used for gene therapy is autologous to the subject that is treated.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding a CRPI is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem or progenitor cells which can be isolated and maintained *in vitro* can be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Direct injection of a DNA coding for a CRPI may also be performed according to, for example, the techniques described in United States Patent No. 5,589,466. These techniques involve the injection of "naked DNA", *i.e.*, isolated DNA molecules in the absence of liposomes, cells, or any other material besides a suitable carrier. The injection of DNA encoding a protein and operably linked to a suitable promoter results in the production of the protein in cells near the site of injection and the elicitation of an immune response in the subject to the protein encoded by the injected DNA. In a preferred embodiment, naked DNA comprising (a) DNA encoding a CRPI and (b) a promoter are injected into a subject to elicit an immune response to the CRPI.

5.14.3 Inhibition of CRPIs to Treat Cardiac Response

In one embodiment of the invention, cardiac response is treated or prevented by administration of a compound that antagonizes (inhibits) the level(s) and/or

function(s) of one or more CRPIs which are elevated in the blood of subjects having cardiac response as compared with blood of subjects free from cardiac response. Compounds useful for this purpose include but are not limited to anti-CRPI antibodies (and fragments and derivatives containing the binding region thereof), CRPI antisense or ribozyme nucleic acids, and nucleic acids encoding dysfunctional CRPIs that are used to "knockout" endogenous CRPI function by homologous recombination (see, *e.g.*, Capecchi, 1989, *Science* 244:1288-1292). Other compounds that inhibit CRPI function can be identified by use of known *in vitro* assays, *e.g.*, assays for the ability of a test compound to inhibit binding of a CRPI to another protein or a binding partner, or to inhibit a known CRPI function. Preferably such inhibition is assayed *in vitro* or in cell culture, but genetic assays may also be employed. The Preferred Technology can also be used to detect levels of the CRPI before and after the administration of the compound. Preferably, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific compound and whether its administration is indicated for treatment of the affected tissue, as described in more detail below.

In a specific embodiment, a compound that inhibits a CRPI function is administered therapeutically or prophylactically to a subject in whom an increased blood level or functional activity of the CRPI (*e.g.*, greater than the normal level or desired level) is detected as compared with blood of subjects free from cardiac response or a predetermined reference range. Methods standard in the art can be employed to measure the increase in a CRPI level or function, as outlined above. Preferred CRPI inhibitor compositions include small molecules, i.e., molecules of 1000 daltons or less. Such small molecules can be identified by the screening methods described herein.

5.14.4 Antisense Regulation of CRPIs

In a specific embodiment, CRPI expression is inhibited by use of CRPI antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least six nucleotides that are antisense to a gene or cDNA encoding a CRPI or a portion thereof. As used herein, a CRPI "antisense" nucleic acid refers to a nucleic acid capable of hybridizing by virtue of some sequence complementarity to a portion of an RNA (preferably mRNA) encoding a CRPI. The antisense nucleic acid may be complementary to a coding and/or noncoding region of an mRNA encoding a CRPI. Such antisense

nucleic acids have utility as compounds that inhibit CRPI expression, and can be used in the treatment or prevention of cardiac response.

The antisense nucleic acids of the invention are double-stranded or single-stranded oligonucleotides, RNA or DNA or a modification or derivative thereof, and can be directly administered to a cell or produced intracellularly by transcription of exogenous, introduced sequences.

The invention further provides pharmaceutical compositions comprising an effective amount of the CRPI antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention provides methods for inhibiting the expression of a CRPI nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising a CRPI antisense nucleic acid of the invention.

CRPI antisense nucleic acids and their uses are described in detail below.

5.14.5 CRPI Antisense Nucleic Acids

The CRPI antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides ranging from 6 to about 50 oligonucleotides. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof and can be single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appended groups such as peptides; agents that facilitate transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, Proc. Natl. Acad. Sci. USA 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988); hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a CRPI antisense oligonucleotide is provided, preferably of single-stranded DNA. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The CRPI antisense oligonucleotide may comprise at least one of the following modified base moieties: 5-fluorouracil, 5-bromouracil, 5-chlorouracil,

5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine, and other base analogs.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, *e.g.*, one of the following sugar moieties: arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one of the following modified phosphate backbones: a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, a formacetal, or an analog of formacetal.

In yet another embodiment, the oligonucleotide is an -anomeric oligonucleotide. An -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), and methylphosphonate oligonucleotides can be

prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:7448-7451).

In a specific embodiment, the CRPI antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the CRPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the CRPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Examples of such promoters are outlined above.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a gene encoding a CRPI, preferably a human gene encoding a CRPI. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize under stringent conditions (*e.g.*, highly stringent conditions comprising hybridization in 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C and washing in 0.1xSSC/0.1% SDS at 68°C, or moderately stringent conditions comprising washing in 0.2xSSC/0.1% SDS at 42°C) with the RNA, forming a stable duplex; in the case of double-stranded CRPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding a CRPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.14.6 Therapeutic Use of CRPI Antisense Nucleic Acids

The CRPI antisense nucleic acids can be used to treat or prevent cardiac response when the target CRPI is overexpressed in the blood of subjects suspected of having or suffering from cardiac response. In a preferred embodiment, a single-stranded DNA antisense CRPI oligonucleotide is used.

Cell types which express or overexpress RNA encoding a CRPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, astrocytes, glial cells, neuronal cells, and ependymal cells). Such methods include, but are not limited to, hybridization with a CRPI-specific nucleic acid (*e.g.*, by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into a CRPI, immunoassay, etc. In a preferred aspect, primary tissue from a subject can be assayed for CRPI expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of a CRPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a subject having cardiac response.

The amount of CRPI antisense nucleic acid which will be effective in the treatment of cardiac response can be determined by standard clinical techniques.

In a specific embodiment, pharmaceutical compositions comprising one or more CRPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, such compositions may be used to achieve sustained release of the CRPI antisense nucleic acids.

5.14.7 Inhibitory Ribozyme andTriple Helix Approaches

In another embodiment, symptoms of cardiac response may be ameliorated by decreasing the level of a CRPI or CRPI activity by using gene sequences encoding the CRPI in conjunction with well-known gene "knock-out," ribozyme or triple helix methods to decrease gene expression of a CRPI. In this approach ribozyme or triple helix molecules are used to modulate the activity, expression or synthesis of the gene encoding the CRPI, and thus to ameliorate the symptoms of cardiac response. Such molecules may be designed to reduce or inhibit expression of a mutant or non-mutant target gene. Techniques for the production and use of such molecules are well known to those of skill in the art.

Ribozyme molecules designed to catalytically cleave gene mRNA transcripts encoding a CRPI can be used to prevent translation of target gene mRNA and, therefore, expression of the gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding a CRPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585-591, each of which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA encoding the CRPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug, et al., 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target

RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the CRPI.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the CRPI *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the CRPI and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficacy.

Endogenous CRPI expression can also be reduced by inactivating or "knocking out" the gene encoding the CRPI, or the promoter of such a gene, using targeted homologous recombination (*e.g.*, see Smithies, et al., 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989, Cell 5:313-321; and Zijlstra et al., 1989, Nature 342:435-438, each of which is incorporated by reference herein in its entirety). For example, a mutant gene encoding a non-functional CRPI (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene encoding the CRPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, the endogenous expression of a gene encoding a CRPI can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene encoding the CRPI in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6),

569-584; Helene, et al., 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bioassays 14(12), 807-815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC^+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) or translation (antisense, ribozyme) of mRNA produced by normal gene alleles of a CRPI that the situation may arise wherein the concentration of CRPI present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of activity of a gene encoding a CRPI are maintained, gene therapy may be used to introduce into cells nucleic acid molecules that encode and express the CRPI that exhibit normal gene activity and that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the gene encodes an extracellular protein, normal CRPI can be co-administered in order to maintain the requisite level of CRPI activity.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.15 Assays For Therapeutic Or Prophylactic Compounds

The present invention also provides assays for use in drug discovery in order to identify or verify the efficacy of compounds for treatment or prevention of cardiac response. Test compounds can be assayed for their ability to restore CRF or CRPI levels in a subject having cardiac response towards levels found in subjects free from cardiac response or to produce similar changes in experimental animal models of cardiac response. Compounds able to restore CRF or CRPI levels in a subject having cardiac response towards levels found in subjects free from cardiac response or to produce similar changes in experimental animal models of cardiac response can be used as lead compounds for further drug discovery, or used therapeutically. CRF and CRPI expression can be assayed by the Preferred Technology, immunoassays, gel electrophoresis followed by visualization, detection of CRPI activity, or any other method taught herein or known to those skilled in the art. Such assays can be used to screen candidate drugs, in clinical monitoring or in drug development, where abundance of a CRF or CRPI can serve as a surrogate marker for clinical disease.

In various specific embodiments, *in vitro* assays can be carried out with cells representative of cell types involved in a subject's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used It is also apparent to the skilled artisan that, based upon the present disclosure, transgenic animals can be produced

with "knock-out" mutations of the gene or genes encoding one or more CRPIs. A "knock-out" mutation of a gene is a mutation that causes the mutated gene to not be expressed, or expressed in an aberrant form or at a low level, such that the activity associated with the gene product is nearly or entirely absent. Preferably, the transgenic animal is a mammal, more preferably, the transgenic animal is a mouse.

In one embodiment, test compounds that modulate the expression of a CRPI are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for cardiac response, expressing the CRPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of the test compound on expression of one or more CRPIs is determined. A test compound that alters the expression of a CRPI (or a plurality of CRPIs) can be identified by comparing the level of the selected CRPI or CRPIs (or mRNA(s) encoding the same) in an animal or group of animals treated with a test compound with the level of the CRPI(s) or mRNA(s) in an untreated animal or group of animals or an animal or group of animals treated with a control compound. Techniques known to those of skill in the art can be used to determine the mRNA and protein levels, for example, *in situ* hybridization. The animals may or may not be sacrificed to assay the effects of a test compound.

In another embodiment, test compounds that modulate the activity of a CRPI or a biologically active portion thereof are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for cardiac response, expressing the CRPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of a test compound on the activity of a CRPI is determined. A test compound that alters the activity of a CRPI (or a plurality of CRPIs) can be identified by assaying animals treated with a control compound and animals treated with the test compound. The activity of the CRPI can be assessed by detecting induction of a cellular second messenger of the CRPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the CRPI or binding partner thereof, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a CRPI of the invention operably linked to a nucleic acid encoding a detectable marker, such as luciferase or green fluorescent protein), or detecting a cellular response (*e.g.*, cellular differentiation or cell proliferation). Techniques known to those of skill in the art can be utilized to detect changes in the activity of a

CRPI (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference).

In yet another embodiment, test compounds that modulate the level or expression of a CRPI (or plurality of CRPIs) are identified in human subjects having cardiac response, preferably those having cardiac response and most preferably those having severe cardiac response. In accordance with this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on CRPI expression is determined by analyzing the expression of the CRPI or the mRNA encoding the same in a biological sample (*e.g.*, blood, serum, plasma, or urine). A test compound that alters the expression of a CRPI can be identified by comparing the level of the CRPI or mRNA encoding the same in a subject or group of subjects treated with a control compound to that in a subject or group of subjects treated with a test compound. Alternatively, alterations in the expression of a CRPI can be identified by comparing the level of the CRPI or mRNA encoding the same in a subject or group of subjects before and after the administration of a test compound. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression. For example, the Preferred Technology described herein can be used to assess changes in the level of a CRPI.

In another embodiment, test compounds that modulate the activity of a CRPI (or plurality of CRPIs) are identified in human subjects having cardiac response, preferably those having cardiac response and most preferably those with severe cardiac response. In this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on the activity of a CRPI is determined. A test compound that alters the activity of a CRPI can be identified by comparing biological samples from subjects treated with a control compound to samples from subjects treated with the test compound. Alternatively, alterations in the activity of a CRPI can be identified by comparing the activity of a CRPI in a subject or group of subjects before and after the administration of a test compound. The activity of the CRPI can be assessed by detecting in a biological sample (*e.g.*, blood, serum, plasma, or urine) induction of a cellular signal transduction pathway of the CRPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), catalytic or enzymatic activity of the CRPI or a binding partner thereof, or a cellular response, for example, cellular differentiation, or cell proliferation. Techniques known to those of skill in the art can be used to detect changes in the induction of a

second messenger of a CRPI or changes in a cellular response. For example, RT-PCR can be used to detect changes in the induction of a cellular second messenger.

In a preferred embodiment, a test compound that changes the level or expression of a CRPI towards levels detected in control subjects (*e.g.*, humans free from cardiac response) is selected for further testing or therapeutic use. In another preferred embodiment, a test compound that changes the activity of a CRPI towards the activity found in control subjects (*e.g.*, humans free from cardiac response) is selected for further testing or therapeutic use.

In another embodiment, test compounds that reduce the severity of one or more symptoms associated with cardiac response are identified in human subjects having cardiac response, preferably subjects having cardiac response and most preferably subjects with severe cardiac response. In accordance with this embodiment, a test compound or a control compound is administered to the subjects, and the effect of a test compound on one or more symptoms of cardiac response is determined. A test compound that reduces one or more symptoms can be identified by comparing the subjects treated with a control compound to the subjects treated with the test compound. Techniques known to physicians familiar with cardiac response can be used to determine whether a test compound reduces one or more symptoms associated with cardiac response. For example, a test compound that reduces aetherosclerosisor vasculitis in a subject having cardiac response will be beneficial for treating subjects having cardiac response.

In a preferred embodiment, a test compound that reduces the severity of one or more symptoms associated with cardiac response in a human having cardiac response is selected for further testing or therapeutic use.

5.16 Therapeutic and Prophylactic Compositions and Their Use

The invention provides methods of treatment (and prophylaxis) comprising administering to a subject an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration are described below.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection into blood or at the site (or former site) of cardiac response.

In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the endothelial cells lining the blood vessels, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such

as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of cardiac response can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

6. EXAMPLE: IDENTIFICATION OF PROTEINS DIFFERENTIALLY EXPRESSED IN THE BLOOD IN CARDIAC RESPONSE

Using the following procedure, proteins in blood samples from control animals and animals having cardiac response were separated by isoelectric focusing followed by SDS-PAGE and analyzed. Serial samples were taken over time. Parts

6.1.2 to 6.1.15 (inclusive) of the procedure set forth are hereby designated as the "Reference Protocol"

6.1 MATERIALS AND METHODS

6.1.1. Induction of Cardiac Response

Cardiac response was induced by treatment with doxorubicin (DX) 1mg/kg either alone or 30 min after an i.p. injection of dexrazoxane (ICRF) 50 mg/kg as described in the Examples *infra.* Treatment with ICRF alone was also administered. The treatment comprised of 7 weekly injections and samples were taken within 24h after the final injection.

Doxorubicin is a marketed anthracycline anticancer drug that causes a serious chronic cardiac toxicity. The spontaneous hypertensive rat is a useful animal model to study this toxicity. Furthermore by incorporating a cardioprotectant in the study design, the association of protein markers with the undesirable cardiotoxicity of the cardiotoxic agent is strengthened. Pretreatment with the intracellular iron chelating compound dexrazoxane (also referred to as ICRF-187) provides significant cardioprotection against clinical doxorubicin-induced cardiotoxicity and the drug has been FDA approved for this indication.

Samples from animals treated to induce cardiac reponse or treated with a cardioprotectant can be collected, prepared and analysed as described herein. In the studies provided herein, animals were administered an intravenous injection of either (i) doxorubicin 1mg/kg alone; (ii) doxorubicin 1mg/kg 30 min after an i.p. injection of dexrazoxane 50 mg/kg or (iii) treated with dexrazoxane 50 mg/kg alone. Control groups received saline only. 7 weekly injections were administered and samples were collected 24h after the final injection. Plasma was collected for discovery of novel biomarkers of cardiac damage using 2-D gel electrophoresis and for measurement of cardiac specific troponin T levels. Tissues were collected for histopathological confirmation of cardiac tissue injury.

6.1.2 Sample Preparation

A protein assay (Pierce BCA Cat # 23225) was performed on each serum sample as received. Prior to protein separation, each sample was processed for

selective depletion of certain proteins, in order to enhance and simplify protein separation and facilitate analysis by removing proteins that may interfere with or limit analysis of proteins of interest. *See* International Patent Application No. PCT/GB99/01742, filed June 1, 1999, which is incorporated by reference in its entirety, with particular reference to the experimental protocol.

Removal of albumin, haptoglobin, transferrin and immunoglobin G (IgG) from blood ("blood depletion") was achieved by an affinity chromatography purification step in which the sample was passed through a series of 'Hi-Trap' columns containing immobilized antibodies for selective removal of albumin, haptoglobin and transferrin, and protein G for selective removal of immunoglobin G. Two affinity columns in a tandem assembly were prepared by coupling antibodies to protein G-sepharose contained in Hi-Trap columns (Protein G-Sepharose Hi-Trap columns (1 ml) Pharmacia Cat. No. 17-0404-01). This was done by circulating the following solutions sequentially through the columns: (1) Dulbecco's Phosphate Buffered Saline (Gibco BRL Cat. No. 14190-094); (2) concentrated antibody solution; (3) 200 mM sodium carbonate buffer, pH 8.35; (4) cross-linking solution (200 mM sodium carbonate buffer, pH 8.35, 20 mM dimethylpimelimidate); and (5) 500 mM ethanolamine, 500 mM NaCl. A third (un-derivatised) protein G Hi-Trap column was then attached to the lower end of the tandem column assembly.

The chromatographic procedure was automated using an Akta Fast Protein Liquid Chromatography (FPLC) System such that a series of up to seven runs could be performed sequentially. The samples were passed through the series of 3 Hi-Trap columns in which the affinity chromatography media selectively bind the above proteins thereby removing them from the sample. Fractions (typically 3 ml per tube) were collected of unbound material ("Flowthrough fractions") that eluted through the column during column loading and washing stages and of bound proteins ("Bound/Eluted fractions") that were eluted by step elution with Immunopure Gentle Ag/Ab Elution Buffer (Pierce Cat. No. 21013). The eluate containing unbound material was collected in fractions which were pooled, desalted/concentrated by centrifugal ultrafiltration and stored to await further analysis by 2D PAGE.

A volume of depleted blood containing approximately 300 g of total protein was aliquoted and an equal volume of 10% (w/v) SDS (Fluka 71729), 2.3% (w/v) dithiothreitol (BDH 443852A) was added. The sample was heated at 95°C for 5 mins, and then allowed to cool to 20°C . 125 l of the following buffer was then added to the sample:

8M urea (BDH 452043w)

4% CHAPS (Sigma C3023)

65mM dithiotheitol (DTT)

2% (v/v) Resolytes 3.5-10 (BDH 44338 2x)

This mixture was vortexed, and centrifuged at 13000 rpm for 5 mins at 15°C, and the supernatant was analyzed by isoelectric focusing.

6.1.3 Isoelectric Focusing

Isoelectric focusing (IEF), was performed using the Immobiline® DryStrip Kit (Pharmacia BioTech), following the procedure described in the manufacturer's instructions, *see* Instructions for Immobiline® DryStrip Kit, Pharmacia, # 18-1038-63, Edition AB (incorporated herein by reference in its entirety). Immobilized pH Gradient (IPG) strips (18cm, pH 3-10 non-linear strips; Pharmacia Cat. # 17-1235-01) were rehydrated overnight at 20°C in a solution of 8M urea, 2% (w/v) CHAPS, 10mM DTT, 2% (v/v) Resolytes 3.5-10, as described in the Immobiline DryStrip Users Manual. For IEF, 50 l of supernatant (prepared as above) was loaded onto a strip, with the cup-loading units being placed at the basic end of the strip. The loaded gels were then covered with mineral oil (Pharmacia 17-3335-01) and a voltage was immediately applied to the strips according to the following profile, using a Pharmacia EPS3500XL power supply (Cat 19-3500-01):

Initial voltage = 300V for 2 hrs

Linear Ramp from 300V to 3500V over 3hrs

Hold at 3500V for 19hrs

For all stages of the process, the current limit was set to 10mA for 12 gels, and the wattage limit to 5W. The temperature was held at 20°C throughout the run.

6.1.4 Gel Equilibration and SDS-PAGE

After the final 19hr step, the strips were immediately removed and immersed for 10 mins at 20°C in a first solution of the following composition: 6M urea; 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol (Fluka 49767); 0.05M Tris/HCl, pH 6.8 (Sigma Cat T-1503). The strips were removed from the first solution and

immersed for 10 mins at 20°C in a second solution of the following composition: 6M urea; 2% (w/v) iodoacetamide (Sigma I-6125); 2% (w/v) SDS; 30% (v/v) glycerol; 0.05M Tris/HCl, pH 6.8. After removal from the second solution, the strips were loaded onto supported gels for SDS-PAGE according to Hochstrasser et al., 1988, Analytical Biochemistry 173: 412-423 (incorporated herein by reference in its entirety), with modifications as specified below.

6.1.5 Preparation of supported gels

The gels were cast between two glass plates of the following dimensions: 23cm wide x 24cm long (back plate); 23cm wide x 24cm long with a 2cm deep notch in the central 19cm (front plate). To promote covalent attachment of SDS-PAGE gels, the back plate was treated with a 0.4% solution of gamma-methacryl-oxypropyltrimethoxysilane in ethanol (BindSilane™; Pharmacia Cat. # 17-1330-01). The front plate was treated with (RepelSilane™ Pharmacia Cat. # 17-1332-01) to reduce adhesion of the gel. Excess reagent was removed by washing with water, and the plates were allowed to dry. At this stage, both as identification for the gel, and as a marker to identify the coated face of the plate, an adhesive bar-code was attached to the back plate in a position such that it would not come into contact with the gel matrix.

The dried plates were assembled into a casting box with a capacity of 13 gel sandwiches. The top and bottom plates of each sandwich were spaced by means of 1mm thick spacers, 2.5 cm wide. The sandwiches were interleaved with acetate sheets to facilitate separation of the sandwiches after gel polymerization. Casting was then carried out according to Hochstrasser et al., *op. cit.*

A 9-16% linear polyacrylamide gradient was cast, extending up to a point 2cm below the level of the notch in the front plate, using the Angelique gradient casting system (Large Scale Biology). Stock solutions were as follows. Acrylamide (40% in water) was from Serva (Cat. # 10677). The cross-linking agent was PDA (BioRad 161-0202), at a concentration of 2.6% (w/w) of the total starting monomer content. The gel buffer was 0.375M Tris/HCl, pH 8.8. The polymerization catalyst was 0.05% (v/v) TEMED (BioRad 161-0801), and the initiator was 0.1% (w/v) APS (BioRad 161-0700). No SDS was included in the gel and no stacking gel was used. The cast gels were allowed to polymerize at 20°C overnight, and then stored at 4°C in sealed polyethylene bags with 6ml of gel buffer, and were used within 4 weeks.

6.1.6 SDS-PAGE

A solution of 0.5% (w/v) agarose (Fluka Cat 05075) was prepared in running buffer (0.025M Tris, 0.198M glycine (Fluka 50050), 1% (w/v) SDS, supplemented by a trace of bromophenol blue). The agarose suspension was heated to 70_C with stirring, until the agarose had dissolved. The top of the supported 2nd D gel was filled with the agarose solution, and the equilibrated strip was placed into the agarose, and tapped gently with a palette knife until the gel was intimately in contact with the 2nd D gel. The gels were placed in the 2nd D running tank, as described by Amess et al., 1995, Electrophoresis 16: 1255-1267 (incorporated herein by reference in its entirety). The tank was filled with running buffer (as above) until the level of the buffer was just higher than the top of the region of the 2nd D gels which contained polyacrylamide, so as to achieve efficient cooling of the active gel area. Running buffer was added to the top buffer compartments formed by the gels, and then voltage was applied immediately to the gels using a Consort E-833 power supply. For 1 hour, the gels were run at 20mA/gel. The wattage limit was set to 150W for a tank containing 6 gels, and the voltage limit was set to 600V. After 1 hour, the gels were then run at 40mA/gel, with the same voltage and wattage limits as before, until the bromophenol blue line was 0.5cm from the bottom of the gel. The temperature of the buffer was held at 16°C throughout the run. Gels were not run in duplicate.

6.1.7 Staining

Upon completion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol (BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ-Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were stained by complete immersion for 4 hours in a staining solution of Pyridinium, 4-[2-[4-(dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt, prepared by diluting a stock solution of this dye (2mg/ml in DMSO) in 7.5% (v/v) aqueous acetic acid to

give a final concentration of 1.2 mg/l; the staining solution was vacuum filtered through a 0.4 m filter (Duropore) before use.

6.1.8 Imaging of the gel

A computer-readable output was produced by imaging the fluorescently stained gels with the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK) described in section 5.1, *supra*. This scanner has a gel carrier with four integral fluorescent markers (Designated M1, M2, M3, M4) that are used to correct the image geometry and are a quality control feature to confirm that the scanning has been performed correctly.

For scanning, the gels were removed from the stain, rinsed with water and allowed to air dry briefly, and imaged on the Apollo 2. After imaging, the gels were sealed in polyethylene bags containing a small volume of staining solution, and then stored at 4°C.

6.1.9 Digital Analysis of the Data

The data were processed as described in U.S. Patent No. 6,064,754 at Sections 5.4 and 5.5 (incorporated herein by reference), as set forth more particularly below.

The output from the scanner was first processed using the MELANIE® II 2D PAGE analysis program (Release 2.2, 1997, BioRad Laboratories, Hercules, California, Cat. # 170-7566) to autodetect the registration points, M1, M2, M3 and M4; to autocrop the images (*i.e.*, to eliminate signals originating from areas of the scanned image lying outside the boundaries of the gel, *e.g.* the reference frame); to filter out artifacts due to dust; to detect and quantify features; and to create image files in GIF format. Features were detected using the following parameters:

Smooths =2

Laplacian threshold 50

Partials threshold 1

Saturation = 100

Peakedness = 0

Minimum Perimeter = 10

6.1.10 Assignment of pI and MW Values

Landmark identification was used to determine the pI and MW of features detected in the images. Thirteen landmark features, designated F1 to F13, were identified in a standard blood image obtained from a pooled sample. These landmark features are identified in Figure 2 and were assigned the pI and/or MW values identified in Table XI.

Table XI. Landmark Features Used In This Study

Name	pI	MW (Da)	Name	pI	MW (Da)
F1	-1	32208	F7	5.31	133062
F2	4.98	57602	F8	-1	10505
F3	5.64	49312	F9	-1	15241
F4	6.35	18635	F10	9.25	25534
F5	5.92	36216	F11	6.06	66164
F6	7.16	74162	F12	4.56	-1
			F13	4.22	12236

As many of these landmarks as possible were identified in each gel image of the dataset. Each feature in the study gels was then assigned a pI value by linear interpolation or extrapolation (using the MELANIE®-II software) to the two nearest landmarks, and was assigned a MW value by linear interpolation or extrapolation (using the MELANIE®-II software) to the two nearest landmarks.

6.1.11 Matching With Primary Master Image

Images were edited to remove gross artifacts such as dust, to reject images which had gross abnormalities such as smearing of protein features, or were of too low a loading or overall image intensity to allow identification of more than the most intense features, or were of too poor a resolution to allow accurate detection of features. Images were then compared by pairing with one common image from the whole sample set. This common image, the "primary master image", was selected on the basis of protein load (maximum load consistent with maximum feature

detection), and general image quality. Additionally, the primary master image was chosen to be an image which appeared to be generally representative of all those to be included in the analysis. (This process by which a primary master gel was judged to be representative of the study gels was rechecked by the method described below and in the event that the primary master gel was seen to be unrepresentative, it was rejected and the process repeated until a representative primary master gel was found).

Each of the remaining study gel images was individually matched to the primary master image such that common protein features were paired between the primary master image and each individual study gel image as described below.

6.1.12 Cross-matching Between Samples

To facilitate statistical analysis of large numbers of samples for purposes of identifying features that are differentially expressed, the geometry of each study gel was adjusted for maximum alignment between its pattern of protein features, and that of the primary master, as follows. Each of the study gel images was individually transformed into the geometry of the primary master image using a multi-resolution warping procedure. This procedure corrects the image geometry for the distortions brought about by small changes in the physical parameters of the electrophoresis separation process from one sample to another. The observed changes are such that the distortions found are not simple geometric distortions, but rather a smooth flow, with variations at both local and global scale.

The fundamental principle in multi-resolution modeling is that smooth signals may be modeled as an evolution through 'scale space', in which details at successively finer scales are added to a low resolution approximation to obtain the high resolution signal. This type of model is applied to the flow field of vectors (defined at each pixel position on the reference image) and allows flows of arbitrary smoothness to be modeled with relatively few degrees of freedom. Each image is first reduced to a stack, or pyramid, of images derived from the initial image, but smoothed and reduced in resolution by a factor of 2 in each direction at every level (Gaussian pyramid) and a corresponding difference image is also computed at each level, representing the difference between the smoothed image and its progenitor (Laplacian pyramid). Thus the Laplacian images represent the details in the image at different scales.

To estimate the distortion between any 2 given images, a calculation was performed at level 7 in the pyramid (i.e. after 7 successive reductions in resolution). The Laplacian images were segmented into a grid of 16x16 pixels, with 50% overlap between adjacent grid positions in both directions, and the cross correlation between corresponding grid squares on the reference and the test images was computed. The distortion displacement was then given by the location of the maximum in the correlation matrix. After all displacements had been calculated at a particular level, they were interpolated to the next level in the pyramid, applied to the test image, and then further corrections to the displacements were calculated at the next scale.

The warping process brought about good alignment between the common features in the primary master image, and the images for the other samples. The MELANIE® II 2D PAGE analysis program was used to calculate and record approximately 500-700 matched feature pairs between the primary master and each of the other images. The accuracy of this program was significantly enhanced by the alignment of the images in the manner described above. To improve accuracy still further, all pairings were finally examined by eye in the MelView interactive editing program and residual recognizably incorrect pairings were removed. Where the number of such recognizably incorrect pairings exceeded the overall reproducibility of the Preferred Technology (as measured by repeat analysis of the same biological sample) the gel selected to be the primary master gel was judged to be insufficiently representative of the study gels to serve as a primary master gel. In that case, the gel chosen as the primary master gel was rejected, and different gel was selected as the primary master gel, and the process was repeated.

All the images were then added together to create a composite master image, and the positions and shapes of all the gel features of all the component images were super-imposed onto this composite master as described below.

Once all the initial pairs had been computed, corrected and saved, a second pass was performed whereby the original (unwarped) images were transformed a second time to the geometry of the primary master, this time using a flow field computed by smooth interpolation of the multiple tie-points defined by the centroids of the paired gel features. A composite master image was thus generated by initializing the primary master image with its feature descriptors. As each image was transformed into the primary master geometry, it was digitally summed pixel by pixel into the composite master image, and the features that had not been paired by the procedure outlined above were likewise added to the composite master image

description, with their centroids adjusted to the master geometry using the flow field correction.

The final stage of processing was applied to the composite master image and its feature descriptors, which now represent all the features from all the images in the study transformed to a common geometry. The features were grouped together into linked sets or "clusters", according to the degree of overlap between them. Each cluster was then given a unique identifying index, the molecular cluster index (MCI).

An MCI identifies a set of matched features on different images. Thus an MCI represents a protein or proteins eluting at equivalent positions in the 2D separation in different samples.

6.1.13 Construction of Profiles

After matching all component gels in the study to the final composite master image, the intensity of each feature was measured and stored. The end result of this analysis was the generation of a digital profile which contained, for each identified feature: 1) a unique identification code relative to corresponding feature within the composite master image (MCI), 2) the x, y coordinates of the features within the gel, 3) the isoelectric point (pI) of the CRFs, 4) the apparent molecular weight (MW) of the CRFs, 5) the signal value, 6) the standard deviation for each of the preceding measurements, and 7) a method of linking the MCI of each feature to the master gel to which this feature was matched. By virtue of a Laboratory Information Management System (LIMS), this MCI profile was traceable to the actual stored gel from which it was generated, so that proteins identified by computer analysis of gel profile databases could be retrieved. The LIMS also permitted the profile to be traced back to an original sample or patient.

6.1.14 Statistical Analysis of the Profiles

Comparisons were made between samples taken from control subjects and samples taken from subjects having cardiac response induced by treatment with (i) doxorubicin (DX), (ii) doxorubicin and dexrazoxane (ICRF) or (iii) dexrazoxane alone.

The statistical strategy used to identify CRFs from the MCIs within the mastergroup is based on the pair-wise student t-test for the average normalized protein abundances between each set of controls and treated samples. The MCIs which

recorded a p-value less than or equal to 0.05 were selected as statistically significant CRFs with 95 % selectivity. Furthermore, a fold change representing the ratio of average abundances between each sample set was calculated for these statistically significant CRFs, and CRFs with fold changes 2.0 were retained.

6.1.15 Recovery and analysis of selected proteins

Proteins in CRFs were robotically excised and processed to generate tryptic digest peptides. Tryptic peptides were analyzed by mass spectrometry using a PerSeptive Biosystems Voyager- DE™ STR Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer, and selected tryptic peptides were analyzed by tandem mass spectrometry (MS/MS) using a Micromass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass, Altrincham, U.K.) equipped with a nanoflow™ electrospray Z-spray source. For partial amino acid sequencing and identification of CRPIs uninterpreted tandem mass spectra of tryptic peptides were searched using the SEQUEST search program (Eng et al., 1994, J. Am. Soc. Mass Spectrom. 5:976-989), version v.C.1. Criteria for database identification included: the cleavage specificity of trypsin; the detection of a suite of a, b and y ions in peptides returned from the database, and a mass increment for all Cys residues to account for carbamidomethylation. The database searched was database constructed of protein entries in the non-redundant database held by the National Centre for Biotechnology Information (NCBI) which is accessible at http://www.ncbi.nlm.nih.gov/. Following identification of proteins through spectral-spectral correlation using the SEQUEST program, masses detected in MALDI-TOF mass spectra were assigned to tryptic digest peptides within the proteins identified. In cases where no amino acid sequences could be identified through searching with uninterpreted MS/MS spectra of tryptic digest peptides using the SEQUEST program, tandem mass spectra of the peptides were interpreted manually, using methods known in the art. (In the case of interpretation of low-energy fragmentation mass spectra of peptide ions see Gaskell et al., 1992, Rapid Commun. Mass Spectrom. 6:658-662)

6.1.16 Results

The severity of cardiac myocyte histopathologic lesions (cytoplasmic vacuolization and myofibrillar loss) was graded semiquantitatively by light microscopy on a scale of 0 to 3. Cardiac lesions were observed in all treated groups

given doxorubicin alone (average lesion score of 2.1) versus saline or dexrazoxane treated groups (average lesion score of 0). Cardiac lesions were attenuated by dexrazoxane pretreatment prior to doxorubicin dosing (average lesion score of 1.0). Plasma cardiac troponin T concentrations were measured by chemiluminescent immunoassay. Terminal cardiac troponin T plasma levels were significantly lower in rats given dezrazoxane plus doxorubicin than in those receiving doxorubicin alone (0.01 versus 0.13 ng/ml). Mean cardiac troponin T plasma levels were below 0.01 ng/ml in dexrazoxane or saline treated animals.

These initial experiments identified 75 features that were decreased and 45 features that were increased in Cardiac Response blood samples as compared with normal blood samples. 4 Features that were decreased and 3 features that were increased were identified in blood samples treated with ICRF as compared with normal blood samples.Details of these CRFs are provided in Tables I, II, III and XII. Each CRF was differentially present in blood from treated subjetcs as compared with blood from non-treated subjects. Partial amino acid sequences were determined for all the CRPIs present in these CRFs. Details of these CRPIs are provided in Tables V, VI and VII

Table XII(a) lists the most highly preferred differentially expressed CRFs with a p-value ≤0.001 ; Table XII(b); lists highly preferred differentially expressed CRFs with a p-value ≤0.01 and Table XII(c) lists preferred differentially expressed CRFs with a p-value ≤0.05. FC denoted Fold Change; DX (deoxyrubicin); ICRF (dexrazoxane)

Table XII CRFs differentially expressed in Cardiac Response

Table XII(a)				FC	T-test	FC	T-test	FC	T-test
p-value ≤0.001	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	4	5.2	15,589	-9.20	1.7E-04				
	6	4.6	34,921	-7.77	1.0E-03				
	9	5.2	15,591	-5.72	1.5E-03	-5.59	0.001		
	19	5.7	68,576	-5.12	3.7E-04				

	79	6.8	80,678	-4.51	1.6E-04				
	82	7.6	77,393	-9.06	2.9E-04				
	84	4.8	73,524	-8.60	1.1E-03				
	118	6.5	33,117	-4.82	3.4E-04				
	144			-3.70	3.7E-04				
	146			-4.19	6.2E-04				
	44	5.8	52,589	4.83	1.1E-03	3.88	0.040		
	115	7.1	38,136	3.55	2.3E-04				
	135	7.3	85,176	4.77	1.2E-05				
	145			3.68	4.5E-04				

Table XII(b)				**FC**	**T-test**	**FC**	**T-test**	**FC**	**T-test**
p-value ≤ 0.01	**CRF**	**pI**	**MW (Da)**	**DX vs Control**	**P-Value, DX vs Control**	**DX & ICRF vs Control**	**P-Value, DX & ICRF vs Control**	**ICRF vs Control**	**P-Value, ICRF vs Control**
	2	5.7	12,367	-10.29	0.010				
	10	5	51,899	-6.99	0.007				
	28	5.7	26,409					-2.93	0.004
	69	5.2	54,619	-3.54	0.005				
	90	5.2	67,336	-3.93	0.005				
	92	6.0	63,037	-8.26	0.006				
	92	6	63,037	-8.26	0.006				
	103	5	56,471	-6.02	0.007				
	104	7	53,989	-11.48	0.003				
	130	4.8	14,082	-4.56	0.009				
	142	5.4	105,458	-5.30	0.007				
	147			-4.61	0.002				
	148			-5.11	0.002				
	149			-3.78	0.002				
	150	4.11	47828	-8.39	0.003	-3.88	0.014		
	152			-3.97	0.003				
	153			-4.57	0.004				
	155			-3.84	0.004				
	156	6.89	78237	-3.99	0.005				
	157			-3.54	0.005				
	158			-4.59	0.005				

Table XII(b)				FC	T-test	FC	T-test	FC	T-test
p-value ≤ 0.01	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	160			-6.43	0.006				
	162			-4.37	0.006	-5.04	0.005	-3.47	0.003
	165			-5.52	0.007	-10.77	0.016		
	166			-3.86	0.008				
	47	4.4	11,368	6.81	0.010				
	56	4.9	48,318					3.12	0.004
	58	6.8	34,063	5.43	0.003	4.24	0.075		
	112	7.1	47,499	4.92	0.006				
	122	5.3	24,915	3.98	0.003				
	127	4.8	23,325	5.25	0.006				
	129	5.4	17,765	4.29	0.010				
	131	4.3	11,917	4.28	0.009				
	134	5.1	51,125	7.99	0.009				
	151			3.50	0.003				
	154	7.79	55965	5.39	0.004				
	159			4.04	0.006				
	161			4.58	0.006				
	163			5.15	0.006				
	164	4.51	72286	4.75	0.007				
	167			3.77	0.008				
	168			4.20	0.009				

Table XII(c)				FC	T-test	FC	T-test	FC	T-test
p-value ≤ 0.05	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	1	5.6	37,430	-23.14	0.021				
	3	5.3	12,781	-9.60	0.034	-4.94	0.022		
	5	5.9	67,060	-10.38	0.014				
	7	5.6	15,634	-7.73	0.016				
	8	5.7	99,793	-6.45	0.013				

Table XII(c)				FC	T-test	FC	T-test	FC	T-test
p-value ≤ 0.05	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	11	4.9	52,439	-6.13	0.013				
	12	5.9	47,310	-4.96	0.022				
	13	6.8	38,368	-4.94	0.018				
	14	6.1	39,111	-5.84	0.016				
	15	5.7	54,294	-5.56	0.019	-3.08	0.018		
	17	6.7	51,594	-4.39	0.021	-3.70	0.009		
	18	6.4	76,207	-4.43	0.037				
	20	5.2	31,123	-4.13	0.023				
	21	4.6	36,159	-3.23	0.049	-2.93	0.051	-10.01	0.038
	22	5.7	24,627			-5.93	0.044		
	72	4.7	39,320			-2.88	0.029		
	80	6.6	81,686	-8.21	0.037				
	87	6.2	67,169	-4.28	0.013				
	89	6.1	66,187	-5.93	0.041				
	94	4.7	58,902	-4.30	0.021				
	96	4.7	58,255	-4.64	0.026				
	98	4.8	57,844	-6.72	0.029				
	99	4.9	57,681	-7.97	0.031				
	100	4.7	57,646	-12.10	0.019	-4.52	0.016		
	102	4.9	56,657	-6.24	0.041				
	105	4.9	52,147	-5.01	0.023				
	116	4.4	36,226	-5.78	0.019			-3.18	0.013
	121	7.1	27,232	-3.48	0.045			-4.01	0.040
	140	5.8	166,485	-4.49	0.022				
	141	6.3	107,802	-3.71	0.020				
	143	6.2	103,573	-3.97	0.013				
	171			-8.14	0.017	-4.80	0.008	-5.45	0.014
	173			-16.09	0.022	-3.01	0.038		
	174			-3.75	0.024				
	177			-5.72	0.031				
	178			-6.59	0.031	-5.06	0.035		
	179			-4.60	0.034				
	181			-4.33	0.038				
	183			-3.82	0.046				

Table XII(c)				FC	T-test	FC	T-test	FC	T-test
p-value ≤ 0.05	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	184			-4.59	0.049	-5.29	0.046		
	185			-3.50	0.050				
	186					-4.18	0.032		
	42	4.6	15,989	4.43	0.041				
	43	5.6	41,054	4.74	0.023				
	45	5.3	31,522	5.47	0.031				
	49	6.2	47,119	9.31	0.017				
	51	4.6	23,573	7.36	0.032				
	52	5.4	22,411	11.54	0.012				
	53	5.6	29,885	7.79	0.017				
	54	5.6	37,507	13.22	0.036				
	59	5.8	36,833			4.20	0.048		
	62	7.2	33,555			4.97	0.022		
	66	6.6	34,783	3.02	0.026				
	83	5.8	78,180	11.13	0.018				
	95	4.9	59,635			9.03	0.022		
	113	6.4	46,986	7.95	0.033				
	119	5.8	30,680	3.43	0.027				
	120	4.9	29,904			4.01	0.029		
	124	5	23,549	5.44	0.043				
	128	5.3	21,197	3.61	0.027				
	132	4.5	10,104	22.18	0.015	12.92	0.084		
	136	7.3	95,488	3.59	0.016				
	169			5.55	0.011				
	170			3.82	0.011				
	172			6.21	0.021				
	175			3.63	0.028				
	176			3.64	0.029				
	180			5.44	0.036				
	182			5.63	0.043				
	187							-4.96	0.044
	188							-4.69	0.047
	189							-4.03	0.027
	73	5.4	29,367					2.75	0.047

Table XII(c)				FC	T-test	FC	T-test	FC	T-test
p-value ≤ 0.05	CRF	pI	MW (Da)	DX vs Control	P-Value, DX vs Control	DX & ICRF vs Control	P-Value, DX & ICRF vs Control	ICRF vs Control	P-Value, ICRF vs Control
	190							3.55	0.013

For some preferred CRFs, CRF-2, CRF-10, CRF-28, CRF-47, CRF-56, CRF-58, CRF-69, CRF-90, CRF-92, CRF-92, CRF-103, CRF-104, CRF-112, CRF-122, CRF-127, CRF-129, CRF-130, CRF-131, CRF-134, CRF-142, CRF-147, CRF-148, CRF-149, CRF-150, CRF-151, CRF-152, CRF-153, CRF-154, CRF-155, CRF-156, CRF-157, CRF-158, CRF-159, CRF-160, CRF-161, CRF-162, CRF-163, CRF-164, CRF-165, CRF-166, CRF-167, CRF-168, the difference was highly significant ($p < 0.01$), and for certain highly preferred CRFs, CRF-4, CRF-6, CRF-9, CRF-19, CRF-44, CRF-79, CRF-82, CRF-84, CRF-115, CRF-118, CRF-135, CRF-144, CRF-145, CRF-146, the difference was still more significant ($p < 0.001$).

The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The contents of each reference, patent and patent application cited in this application is hereby incorporated by reference in its entirety.

When a reference is made herein to a method of treating or preventing a disease or condition using a particular agent or combination of agents it is to be understood that such a reference is intended to include the use of that agent or combination of agents in the preparation of a medicament for the treatment or prevention of the disease or condition